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Grapes and Wine

Edited by Antonio Morata,
Iris Loira and Carmen González

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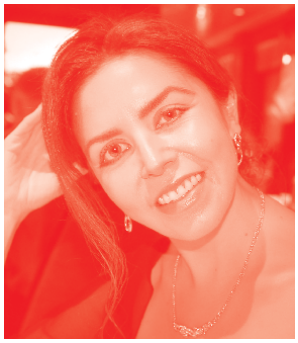

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Contributors
Raúl Ferrer-Gallego, Ana María Mislata, Miquel Puxeu, Montserrat Mestres, Georgiana-Diana Dumitriu (Gabur), Valeriu V. Cotea, Carmen Teodosiu, Agustín Aranda, Emilia Matallana, Cecilia Picazo, Víctor Garrigós, Marius Bumbac, Cristina Mihaela Nicolescu, Cristiana Radulescu, Lavinia Claudia Buruleanu, Radu Lucian Olteanu, Laura Monica Gorghiu, Gabriela Teodorescu, Carmen Georgeta Holban, Luís Filipe-Ribeiro, Fernanda Cosme, Fernando M. Nunes, Eduardo Tapia, Fabiola Altimira, Nancy Vitta, Svetlana Levchenko, Irina Vasylyk, Vladimir Likhovskoy, Vladimir Volynkin, Alla Polulyakh, Ma Tengzhen, Han Shunyu, Ana Cristina Agulheiro-Santos, Sara Ricardo-Rodrigues, Marta Laranjo, Mario de la Fuente, Bartosz Tylkowski, Josep M. M. Montornes, Magdalena Olkiewicz, Ruth Pereira, Anabela Cachada, David Fernández-Calviño, Tito Caffi, Giorgia Fedele, Felicidad De Herralde, Nathaniel K. K. Newlands, Julia Crespo, Juan Mariano Cabellos, Margarita García, Valeria Romero, Teresa Arroyo, Antonio Morata, Iris Loira, Cristian Vaquero, Carlos Escott, Juan Manuel Del Fresno, María Antonia Bañuelos, Carmen González, Felipe Palomero, Carmen López, Javier Raso, Carlota Delso, Alejandro Berzosa, Jorge Sanz, Ignacio Alvarez, Laura Pulcini, Elisa Gamalero, Antonella Costantini, Enrico Tommaso Vaudano, Christos Tsolakis, Emilia Garcia-Moruno, Haluk Hamamci, Nahide Seray Seray Kahraman, Fernando Zamora, Pere Pons-Mercadé, Pol Giménez, Glòria Vilomara, Marta Conde, Antoni Cantos, Nicolas Rozès, Sergi Ferrer, Joan Miquel Canals, Andrii Tarasov, Miguel Cabral, Christophe Loisel, Paulo Lopes, Christoph Schüßler, Rainer Jung

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Meet the editors

Antonio Morata is a Professor of Food Science and Technology at the Universidad Politécnica de Madrid (UPM), Spain, specialized in wine technology. He is a coordinator of the module of enology and winemaking in the Master of Viticulture and Enology, and a coordinator of the Master of Food Engineer at UPM. Dr. Morata is also a professor of enology, and wine technology at the European Master of Viticulture and Enology, Euromaster Vinifera-Erasmus+. He is a Spanish delegate to the group of Experts in wine microbiology of the International Organizations of Vine and Wine (OIV). He authored more than 80 research articles, 2 books, 5 edited books, 6 special issues, and 20 book chapters.

Iris Loira is an Associate Professor of Food Science and Technology at the Universidad Politécnica de Madrid (UPM), Spain, and a professor of the module of enology and winemaking at the Master of Viticulture and Enology. She is an academic secretary for the Master of Food Engineer at UPM. Dr Loira is a professor of wine analysis at the European Master of Viticulture and Enology, Euromaster Vinifera-Erasmus+. She is the author of 50 research articles, 3 books, and 14 book chapters.

Professor González-Chamorro has worked as a professor at the UPM since 1993. She has dedicated her teaching work to food technology and applications in the fruit and vegetable industries and fermented meat products. From 2004 until 2016 she held management positions at the university (Ombudsman and Deputy Director of University extension and International Relations). Her research activity has focused on the field of oenological biotechnology and on the selection of microorganisms (yeasts and BAL) that are of special interest in wine-making processes. She has extensive experience in the use of instrumental and sensory tests to assess the quality of alcoholic beverages (wine and beer) and meat products. She has participated in different educational innovation projects and coordinated three of them. These projects have made it possible to coordinate working groups for the implementation of degrees in the EEES and apply new teaching methodologies that allow the acquisition of horizontal competences by students. She has also evaluated research projects and national and international degrees (different Quality Agencies).

Contents

Preface

Grapes and Wine is my fourth book project with IntechOpen, this time with my colleagues Professors Iris Loira and Carmen González as coeditors. The previous projects (*Grape and Wine Biotechnology*, *Yeasts* and *Advances in Grape and Wine Biotechnology*) have raised a lot of expectations with many downloads and citations (>80kdownloads and >130 WoS citations), and many eminent researchers have participated in them and contributed to their quality. I hope that this new book, also focused on grape and wine sciences, will become a useful tool for vine and wine researchers, professionals and students. I am especially proud of the development of these projects as open access books to ensure that knowledge can be freely accessible to everyone.

The book compiles research and review work on several topics such as grape varieties, pests, biotechnology, winemaking, emerging non-thermal technologies, wine stabilization, off-flavors, and even big data applications. The chapters have been authored by key researchers from nine countries and three continents. *Grape and Wines* is divided into three sections: "Grape Production and Plant Management," "Fermentation and Microbiology," and "Enology and Stabilization." The first section includes chapters on pest control and pesticide management with an integrative perspective from plant to wine. The use of copper in vineyards is also reviewed along with detailed measures to reduce the use of this antifungal product. The use of big data and artificial intelligence is also analyzed in a specific chapter together with their application in grape production and winemaking. The peculiarities of varieties in Romania and Crimean Peninsula are also considered and, lastly, the impact of table grape production is also included.

The second section "Fermentation and Biotechnology" is focused on the use of *Saccharomyces* and non-*Saccharomyces* yeasts in wine fermentation, genetically modified yeast and gene editing to produce yeasts with improved features. This chapter includes gene editing using CRISPR-Cas9 and Synthetic Genome Engineering. The determination of glucose isomerase is used as a tool to prevent sluggish fermentations due to deficient sugar uptake in yeasts. The typical problem of wines from warm areas, such as high pH and neutral aroma, is addressed by using biological acidification with non-*Saccharomyces Lachancea thermotolerans* and the production of fermentative aroma by apiculate yeasts of *Hanseniaspora* spp.

The last section devoted to "Enology and Stabilization" includes the use of innovative non-thermal technologies such as pulsed electric fields for microbial control in grapes and the possibilities of this technology to contribute to the reduction of SO_2 levels in wines. The use of skin contact macerations with terpenic Muscat varieties to improve also the flat aroma of some white wines in warm regions in a climatic change scenario is analyzed. Winemaking in cold areas of northwest China and the peculiarities and problems concerning the difficulties to reach a suitable grape ripeness, especially in long cycle varieties, are detailed in a specific chapter by professors of the Gansu Agricultural University. Wine stability causes several concerns to wine producers and many white wines are affected by protein haze which produces turbidities that disturb the visual appearance of the wines and degrades their quality. The origin and

control of this alteration are discussed in a specific chapter. Light is another physical phenomenon that can affect wine aroma by the formation of riboflavin derivatives that degrade wine quality; the control and management of this alteration are also included. Most of the quality of natural sparkling wines depends on yeast autolysis and the release and formation of molecules with an impact on aroma and flavor. The complex process of autolysis is reviewed and key advices for the best management of this specific biological ageing are included. Lastly, the presence in wines of TCAs off-flavors derived from fungal activity and halogenated precursors is studied, considering the origin, analytical techniques and control measurements.

We hope this new book offers new tools and knowledge that will help students, academics and producers to better understand the technological and biotechnological tools to manage grape production and enhance wine quality.

Antonio Morata, Iris Loira and Carmen González
EnotecUPM,
Department of Chemistry and Food Technology,
ETSIAAB,
Universidad Politécnica de Madrid,
Madrid, Spain

Section 1

Grape Production and Plant Management

Chapter 1

Integrated Pest Management of *Lobesia botrana* with Microorganism in Vineyards: An Alternative for Clean Grapes Production

Fabiola Altimira, Nancy Vitta and Eduardo Tapia

Abstract

The moth *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae) is one of the principal pests of the grapevines (*Vitis vinifera L.*). His larvae feeds from grape, reducing production and increasing susceptibility to fungal infections. This makes it one of the most economically important pest insects in wine and table grape exporting countries. This chapter will describe the distribution, biology, and behavior of *L. botrana* regarding its host, the grapevine, along with its control via the use of natural enemies, entomopathogenic microorganisms, MD (mating disruption) and chemical control. Finally, we will describe an integrated management strategy based on monitoring, MD, and biological control using entomopathogenic microorganisms. This strategy could be useful as a basis for integrated pest control plans in various regions worldwide.

Keywords: *Lobesia botrana* (Denis & Schiffermüller), grapevine, integrated pest management, biological control, ethological control and chemical control

1. Introduction

L. botrana was first scientifically described in 1775 by Denis and Shiffermüller in Austria. This pest is endemic to the Palearctic Region, but is economically more important in southern Europe and South America [1–3]. In Europe it principally affects southern France, central and southern Spain, Portugal, Greece, Italy and the Mediterranean islands [2, 4], while in South America it affects Argentina and Chile [5]. Its broad range is partly attributable to its ability to adapt to climate changes, characteristic of lepidopterans [6] causing a lack of synchronization with its natural parasites and predators and contributing to significant short-term increases in *L. botrana*. Its nature as a polyphagous pest also contributes to its swift establishment in any geographic region it reaches. In its larval stage, it has been reported to eat grapes along with 40 other plant species belonging to 27 families. These host plants generally grow in warm and dry environments, and include *Olea europea* L., *Zizyphus vulgaris* L., *Rosmarinus officinalis* L., *Clematis vitalba* L., *Cornus* spp.,

Lonicera xylosteum L., *Viburnum lantana* L, *Ligustrum vulgare* L., *Ribes* spp., and *Hedera helix* L, among others [7–10]. To develop an integrated *L. botrana* management strategy, we must (1) adequately identify and monitor this pest in its different development stages and its natural enemies. (2) determine the economic damage thresholds at which to begin controlling. (3) Take management decisions according to information from monitoring. (4) Do natural, cultural and biological follow-ups along with the use of selective chemical insecticides, where necessary.

2. Life cycle of *L. botrana* on grapevines

L. botrana is a multivoltine species with a facultative diapause (physiological state of inactivity). The number of generations depends on latitude, photoperiod, humidity, temperature, climate, microclimate and food type [11]. In Europe, two generations per year are common in Germany, Switzerland, Austria and northern France, while three generations (and sometimes four) have been reported in southern France, Spain, Portugal, Greece and Italy [12, 13]. In Chile at least three and possibly four annual generations are known [14].

The eggs of the first generation are deposited separately or in groups of two or three on grapevine buds, pedicels and flowers [15]. Their shape is elliptical, flat and slightly convex, and they measure between 0.65–0.90 mm long by 0.45–0.75 mm wide. Recently laid eggs are translucent and creamy white in color (**Figure 1A**), turning pale yellow with time (**Figure 1B**). They then turn black, with the head of the developing larva visible (**Figure 1C**) [16]. Finally, the egg hatches 7–11 days after laying, depending on temperature and humidity conditions (**Figure 1D**) [8, 15]. Once the larva emerges from the egg, only the shell or the round and nacreous mark of the shell remains (**Figure 1E**).

L. botrana larvae have five development stages (**Figure 2**): I (L1: 0.9–1-0 mm), II (L2: 1.9–3.0 mm), stage III (L3: 4.5–5.0 mm), stage IV (L4: 6.0–7.0 mm) and stage V (L5: 10.0–11.0 mm). Larval development concludes after 20 to 30 days in optimal conditions of 26.7°-29.4°C and 40–70% relative humidity [14].

First generation larvae are called the anthophagous generation, since they attack the plant in or near its flowering season, feeding on flower buttons, flowers and occasional small recently formed fruits. First generation larvae form "nests" or glomerules before and during flowering (**Figure 3**) [14]. These glomerules are formed by various flower buds joined together by silk threads spun by the larvae [8]. Damage caused by first generation larvae on the vines have minimal repercussions [17]. However, larvae in the second generation cause decreased vine productivity, since they attack developing grapes, perforating the skin and feeding on their pulp. Finally, these grapes are scared (**Figure 4**), dry out, fall or rot, depending on their size and the ambient humidity. Third generation larvae, by comparison with second generation larvae (both called carpophagous generations) produce greater damage to vine productivity, since the grapes are matured or in the maturation process [14]. Therefore, larval action exposes their sugary juices, favoring the entry,

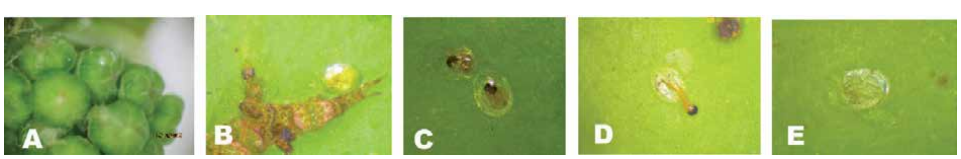

Figure 1.
L. botrana eggs. A, creamy white egg. B, yellow eggs. C, black head egg. D, larva hatching, E) round and nacreous mark.

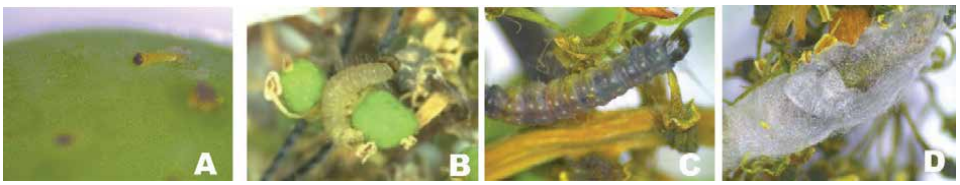

Figure 2.
L. botrana larvae. A, newborn larva. B, young larva. D, mature larva. E, stage V larva spinning a grayish-white silk cocoon for the pupation process.

Figure 3.
L. botrana *glomerules on grape bunches.*

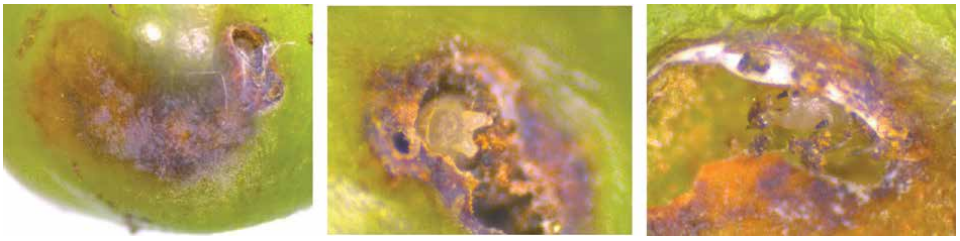

Figure 4.
Grape damage from L. botrana *larvae.*

establishment and proliferation of microorganisms responsible for diseases including *Botrytis cinerea* (Persoon: Fries) (*Sclerotiniaceae)* [18] and black *Aspergillus* (*Aspergilus niger* and *Aspergilus carbonarius*) which produces ochratoxin A [1].

L. botrana pupae are elongated, with a green to dark brown color. The average length of a male and female pupa is 5.5 mm and 7.0 mm, respectively, while the average width is 1.6–1.7 mm (**Figure 5A**). Males have 4 abdominal segments, and

Figure 5.
L. botrana *pupae. A, left – female and right - male. B, pupae in diapause with cocoon.*

females have 3. Their eyes, antennae, wings and abdominal segments can be seen in their structure. Pupae are covered by a silky, white, fused and continuous cocoon.

In vineyards, *L. botrana* hibernates in the pupal stage principally beneath the grapevine bark, in trunk cracks, soil and fallen leaves. During this period the pupae are in diapause, presenting a thick, highly hydrophobic cocoon. This tissue protects the pupa from low temperatures and water (**Figure 5B**) [19]. Full pupal development in diapause takes around 90 days while the pupal state during spring and summer is around 12–14 days, or 130°C days [14, 16].

In springtime, when temperatures rise, adults emerge from pupae in diapause. They emerge in stages, beginning before grapevine budding or extending over several weeks. The first adults to emerge are generally males, but in the later part of the flight period females predominate.

Adult *L. botrana* specimens are 6.0–8.0 mm long with a wingspan of 11.0–13.0 mm. Both sexes have a dorsal design with a cross-sectional band on the front wing pair, which can be seen with the wings laid to rest over the body. Male lack a side fold in their front wings; their back wings are whitish with a brown edge, while female rear wings are completely brown [16]. They can live from one to three weeks. Their activity is crepuscular, remaining inactive during the day and hiding in leaves and bunches. They mate in flight (1 to 6 days after emerging), females generally mate once in their lives. Egg laying begins one or two days after mating, and each female can lay between 80 and 160 eggs [16].

Regarding the dispersion capacity of *L. botrana* moths, males can fly several meters above vegetation and use air currents for longer migrations, while females generally spread over small areas and cannot go beyond 100 m [20]. This indicates that *L. botrana* colonization in new territories occurs mainly due to transferring pest-infested materials.

3. Chemical control

Insecticides are applied according to economic damage level, which can vary depending on generation, cultivar susceptibility to subsequent infection by *B. cinerea* and the grape product target market (wine production or fresh consumption). Chemical control of the first generation is only applied when pest population density reaches 50% of buds infested. The apparent greater flexibility of the damage threshold for controlling the first generation lies in the fact that during the flowering and harvest periods, the reduction of flowers and grapes is compensated by increased size and weight of healthy grapes. For following larval generations, the damage threshold varies between 1% and 5% or between 10% and 15% of bunches damaged, depending on the cultivar, bunch rigidity and harvest time [21].

Neurotoxic insecticides are mainly used for controlling *L. botrana* populations, including chlorantraniliprole, abamectin, indoxacarb, chlorpyrifos, methyl chlorpyrifos, anthranilic diamides, emamectin and spinosad. Growth regulators are also used, including fenoxycarb, methoxyfenozide, and tebufenozide. All the insecticides mentioned are larvicides; however, methoxyfenozide, chlorantraniliprole and indoxacarb are also ovicides.

To be effective, these substances must be applied when the pest is in its most vulnerable development stage, which makes predicting the *L. botrana* development cycle fundamental for determining optimal treatment programs. Selective insecticide programs along with population monitoring via pheromone traps and field monitoring for eggs generally provide adequate *L. botrana* control [22].

4. Ethological control: pheromones and their use in mating disruption (MD)

Pheromones are volatile chemical messengers released into the environment which can influence the behavior of other individuals of the same species at a distance. They are secreted by individuals via their exocrine glands. They are highly specific at the species level, affecting insects' aggregation, dispersion, alarm and sexual behavior [23].

In the exocrine glands of female *L. botrana* specimens, a linear hydrocarbon chain of 15 carbons have been identified which present acetate and alcohols as functional groups. The principal pheromone compound among these is (E, Z) -7,9-dodecadienyl acetate. *L. botrana* can sense and respond to this compound in a wide range of concentrations between 0.1–2500 ng [24, 25].

The chemical attractant capacities of this pheromone lead to its use as a tool for monitoring adult male *L. botrana* specimens. Monitoring is done via counting captured males which are trapped on the sticky surfaces of female pheromone traps (**Figure 6**). Female pheromone use also allows us to control pest populations via MD. This strategy consists of interfering with insects' olfactory chemical communication via mass distribution of synthetic pheromones in the field with MD dispensers. This creates a pheromone cloud which disorients and confuses the males and keeps them from finding females, thereby impeding mating and reducing pest populations [23]. The MD strategy relies on two different mechanisms: one is competition between females and MD dispensers in attracting males; and the other is based on camouflaging the olfactory track which have on females. Commercial MD dispensers, carry the compound (E, Z) -7,9- dodecadienyl acetate, which is progressively sprayed into the farming environment for a determined period of time. The release rate for each unit is generally 50–60 μg/h [26].

When applying this method, pest population density must be considered, as it is more effective with a lower adult population density. Above a certain density, mating is not interrupted regardless of ambient pheromone concentrations; the critical density for *L. botrana* is 4000 couples per hectare, and beyond this population density, the effectiveness of MD drops drastically [20]. Furthermore, when bunches are infested at a rate of 5–10% during the first generation, the effectiveness of MD in following generations is greatly reduced [21, 27, 28].

For MD to be effective, 500 sexual MD dispenser per hectare must be installed in vineyards before the first seasonal flight begins. MD dispenser must be uniformly distributed around the vineyard and attached to shoots so that foliage protects them from direct sunlight exposure and high temperatures [23]. To compensate for atmospheric pheromone dilution around lot perimeters, twice as many MD dispenser must be placed along property edges [29].

Figure 6.
Ethological control. A, traps baited with synthetic lures. B, MD emitter for L. botrana *control.*

MD efficacy evaluation is done by checking the presence of adults and larvae via field monitoring and follow-up. Catching males in traps baited with synthetic lures is considered the easiest way to evaluate MD effectiveness. Capturing no males in traps is considered a "necessary but insufficient" indicator of effective MD, since the pheromone quantity necessary to interrupt males' orientation towards traps baited with synthetic lures is lower than the amount needed to disrupt mating [30]. Thus, capturing a few males in the same trap indicates a high risk of MD control strategy failure. The reliability of traps for monitoring adults might be increased by the use of high-dose lures. In other hand, monitoring of this pest and its damages can be done in the vineyard to determine infestation rate. For this, the following variables must be considered: percentage of bunches infested, number of larvae per inflorescence, number of eggs, larvae and damaged grapes per bunch. The mean number of larvae per bunch gives the most precise evaluation of meting disruption effectiveness, while the number of larvae per inflorescence (i.e., the number of first-generation larvae) can be very quickly evaluated in the field. Precise larval population estimates during the second and third generation require destructive sampling and dissection which take significant time, especially for varietals with compact grape bunches. Sexual confusion evaluations based on final crop damage can be deceptive because this damage, especially primary and secondary rotting, may be due to factors apart from larva feeding [30].

Finally, it must be noted that employing MD has many advantages, including being an ecologically clean method which leaves no wastes, is targeted and does not alter the ecosystem. Finally, it has a cumulative effect through the years, along with being comfortable to apply [23].

5. Biological control: natural enemies

An alternative to chemical control is using natural enemies such as "parasites and predators". Around 21 species have been described as preying on *L. botrana*, belonging to the following orders: Neuroptera, Coleoptera (coccinelids, carabids, clerids, malachiinae), Dermaptera, Hymenoptera, and Hemiptera. In laboratory tests, the predator *Chrisoperla defreitasi* (Neuroptera*:* Chrysopidae) has been observed eating eggs, larvae and pupae of *L. botrana* [23].

97 species of insects can parasitize *L. botrana* [31], belonging to the families Tachinidae (**Figure 7A**), Ichneumonidae, Pteromalidae and Chalcididae, among others. Among the ichneumenoid parasites, *Campoplex capitator* stands out due to its natural efficiency, density and wide geographic distribution. It has been regularly found in most European vineyards (Italy, Spain, Switzerland and France). *C. capitator* parasitizes *L. botrana* pupae in diapause. Freeing them en masse at the start of the season could reduce reproduction of later generations of this pest. *Trichogramma* spp. are microhymenopteras which act on eggs (egg-eating parasites) (**Figure 7B** and **C**). Their action has the advantage of controlling this pest before it can cause harm. In laboratory tests, 95% parasitism has been achieved. Freeing them en masse (thousands of micro-wasps per week) in the field could be useful for egg control. To use these parasites, it is important to monitor adult moths present in the field in order to effectively control eggs. Similarly, Ichneumonidae (**Figure 7D** and **F**) can be a good alternative for controlling *L. botrana*, as they attack larvae and pupae of a wide variety of insects. *Dibrachys affinis* Masi, which belongs to the Pteromalidiae family, also acts upon *L. botrana* chrysalises, reaching parasitism rates of 88%. The ectoparasite *Apanteles* sp. has been noted in the larval stage of *L. botrana.* (**Figure 6**). It has the advantage of global distribution [23].

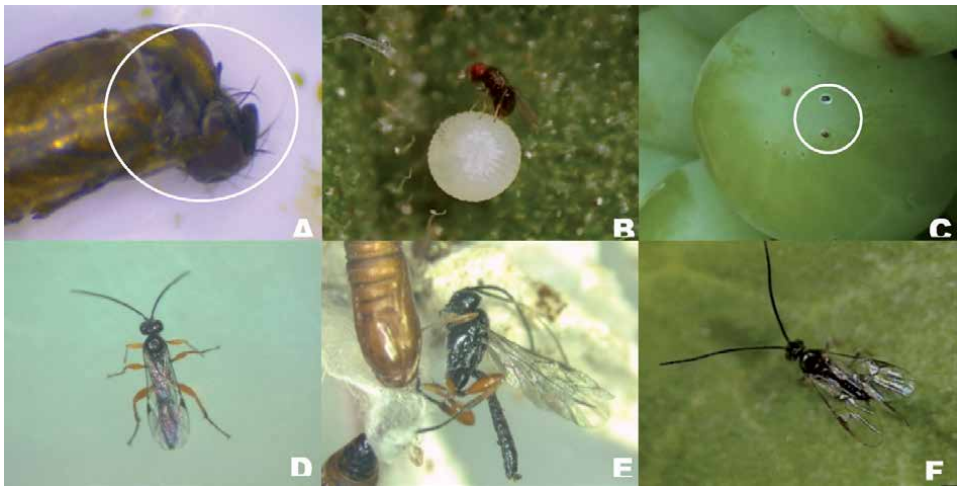

Figure 7.
Natural enemies for controlling L. botrana. *A, adult* Phytomyptera nigrina *(Diptera:Tachinidae) emerging from* L. botrana *pupa. B,* Trichogramma sp. *parasitizing egg. C,* L. botrana *eggs parasitized by* Trichogramma. *D, adult Ichneumonidae. E, adult Ichneumonidae parasitizing* L. botrana *pupa. F, adult* Apanteles *sp.*

6. Biological control: *Bacillus thuringiensis*

Within the biological control market, biopesticides based on *Bacillus thuringiensis* are the most used worldwide due to their toxicity towards a wide range of pest insects from different orders and harmlessness to humans [32].

The insecticidal activity of most *B. thuringiensis* subspecies is due to their producing a cytoplasmic inclusion called δ-endotoxin, which is synthesized during the sporulation process [33]. The δ-endotoxins of the two *B. thuringiensis* subspecies *kurstaki* and *aizawai* are insecticidal against *L. botrana* larvae. This insecticidal action occurs when spores and endotoxins are ingested by the larvae, and then solubilized and turned into active toxins with lower molecular mass by insect proteases in the alkaline pH of larvae midgut. Active toxins bond to specific receptors and induce pore formation in the membrane of intestinal cells, causing membrane integrity loss and cellular lysis that allows bacteria to enter the hemocoel (insect circulatory system), finally leading to larval death due to starvation and sepsis [34]. *L. botrana* larval stage 1 is the most susceptible to δ-endotoxin action, so it is recommended to monitor grape bunches and apply this strategy to eggs in the black head development stage. In this way, emerging L1 larvae will have direct contact with the biopesticide.

The lethality of δ-endotoxins from *Bacillus thuringiensis* groups Cry1, Cry2 and Cry9 which presented activity against Lepidopterae was evaluated on L1 stage *L. botrana* larvae [35]. The toxins with the greatest insecticidal activity were Cry9Ca, Cry2Ab and Cry1Ab, with LC50 values of 0.09, 0.1 and 1.4 μg/ml, respectively. Cry9Ca and Cry1Ab do not share affinity with the same receptor, so combining both δ-endotoxins together with *B. thuringiensis* would allow for better control of L1 stage *L. botrana* larvae [35].

7. Biological control: entomopathogenic fungi

Entomopathogenic fungi (EPF) are microorganisms able to infect and naturally control arthropod populations, allowing them to be used as an alternative to chemical insecticides for pest control. In the microbial pest killer market, around

80% of available EPF products are based on species from the *Metarhizium* and *Beauveria* genera, since both have a wide range of hosts and are easy to mass-produce [36]. *Metarhizium* and *Beauveria* include different species which over time have expanded, due to new types being isolated worldwide and the use of molecular techniques which allow for conclusive and certain identification.

EPF form complex relations with plants, apart from naturally controlling arthropod populations. Studies have shown that EPF species *M. robertsii* and *B. bassiana* provide plants part of the nitrogen which they absorb during insect parasitization [37, 38], promoting plant growth [39]. *Beauveria bassiana* has also been shown to act as an endophyte (colonizing plant interiors) in around 25 plant species, contributing to control of pests and phytopathogenic fungi [38, 40, 41]. It colonizes leaves, buds and roots, allowing plants to be more resistant to insect attacks [38, 42].

The action mechanism developed by EPF to parasitize insects requires EPF to differentiate into morphologically different cellular structures: conidium, germ tube, appressorium, hypha and blastospores. These structures participate in the insect infection and parasitizing process: conidia adhesion to the host cuticle (**Figure 8A**), formation and differentiation of the germinal tube in a structure called appressorium along with its penetration inside the insect cuticle (**Figure 8B**). Hemocoel colonization by blastospores (**Figure 8C**). Emergence of EPF hyphae from inside the insect and EPF sporulation on the corpse (**Figure 8D**), thereby promoting conidia dispersion and the start of new infections.

Although the action mechanism of EPF is known and interest in adopting biological pest control strategies is high, there are few scientific studies which have evaluated EPF effectiveness on *L. botrana* in field conditions. To this end, the study by Cozzi et al. [1] determined the lethality of 6 EPF isolates in an *in vitro* test on *L. botrana* larvae. The best strain, *B. bassiana* ITM 1559, showed a mortality rate of 55% of individuals of this pest. Furthermore, in field tests the incidence of bunches harmed by *L. botrana* larvae was significantly reduced via treatment with this strain, by comparison with the untreated control. In the study by Altimira et al. [19] 100%

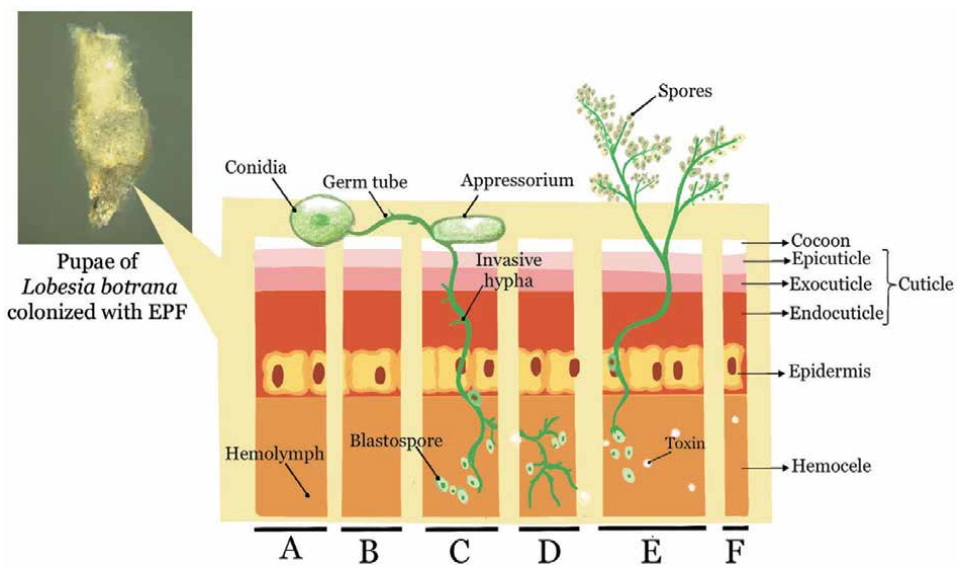

Figure 8.
Infection and development cycle of entomopathogenic fungus (EPF) on an insect pupa. Panel A: Conidium adhesion; panel B: Spore germination; panel C: Appressorium differentiation and cuticle penetration; panel D: Hemocoel colonization; panel E: Hyphae emergence and sporulation; panel F: Strata which EPF must cross to colonize the hemolymph.

effectiveness was obtained against un-cocooned *L. botrana* pupae via using a wettable powder formulation of the strain *B. pseudobassiana* RGM 1747. This field test was done with a controlled infestation of *L. botrana* in ´Red Globe' *V. vinifera* during autumn (average temperature 9.1°C). In natural infestation trials, an effectiveness rate of 51% was achieved in different *V. vinífera* varieties with an average temperature of 8.4°C. During this period, the adhesion, germination and colonization of *B. pseudobassiana* in cocooned pupae was achieved, demonstrating its effectiveness in climate conditions with low temperatures, rain and high humidity present in this time of year in the Metropolitan Region of Chile [19]. Subsequently, Tapia [43] achieved 80% effectiveness with the inverse emulsion formula of the *M. robertsii* RGM 678 strain against *L. botrana* pupae in field tests, along with achieving a significantly lower percentage of male *L. botrana* captures compared to the control treatment.

8. Proposal for integrated *Lobesia botrana* management in Chile

Chile is the main global table grape exporter. One major challenge for grapevine cultivation is controlling *L. botrana*, which has been declared a quarantining pest in this country, due to the economic damages it generates to grapevines and in table grape exportation. The presence of any individual of this species (egg, larva, etc.) on fruit causes the full lot to be rejected for exportation to target markets without *L. botrana*.

In Chile *L. botrana* has three annual generations, with a diapausal pupal state in the autumn-winter period. In this condition *L. botrana* lives under grapevine bark and has a highly hydrophobic cocoon impeding agrochemicals' penetration, making control difficult. However, EPF strains adapted to low temperatures have shown their ability to infect *L. botrana* in this state [19], with greater control efficacy in early autumn [43], since *L. botrana* cocoons in the start of the season are less dense and hydrophobic, facilitating EPF action. Controlling this pest in autumn and winter allows for reducing individuals in the first flight. In spring, we recommend monitoring black head eggs to apply *B. thuringiensis*. Tapia [43] achieved efficacy rates or 55–85% with various commercial products on 'Red Globe' *V. vinifera* crops. The impacts of EPF and *B. thuringiensis* are shown in **Figure 9** [43]. Based on these studies we propose an integrated control program with EPF-based biopesticide applications from early autumn to late winter, complementing these applications with *B. thuringiensis* from early spring to late summer, according to black head egg

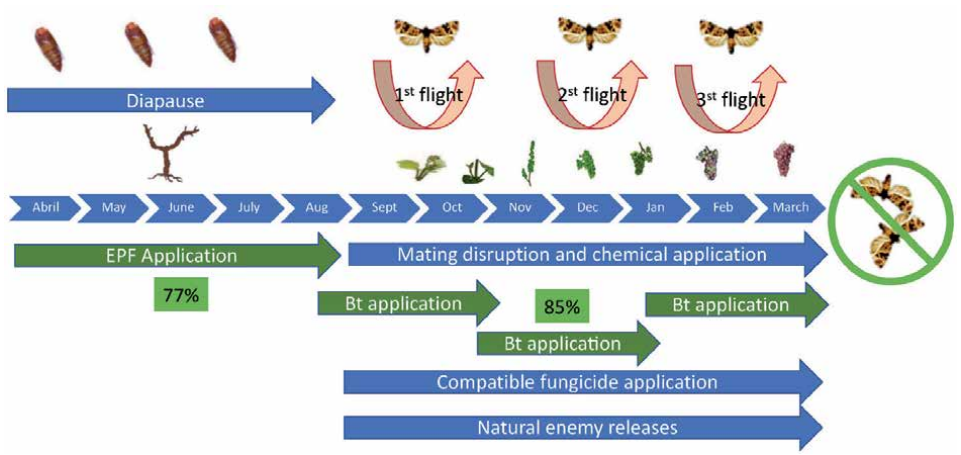

Figure 9.
Integrated Lobesia botrana *management plan with biopesticides and other control tools.*

monitoring. The integrated management plan must consider the MD strategy in vineyards and releasing natural enemies in urban zones with pest concentrations, along with applying synthetic chemical products -preferably green label-after moth flight alerts (**Figure 9**).

9. Conclusion

L. botrana is a pest economically important in southern Europe and South America. Despite the wide host range recorded, grapevine is the major host crop in which damage is really significant. To develop an integrated *L. botrana* management strategy, we must (1) adequately identify and monitor this pest in its different development stages and its natural enemies. (2) determine the economic damage thresholds at which to begin controlling. (3) Take management decisions according to information from monitoring. (4) User different biological tools together with MD allows for reasonable use of synthetic chemical molecules to control *L. botrana*, achieving a sustainable and environmentally friendly production and ultimately, a healthier grape for eating or winemaking.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Fabiola Altimira, Nancy Vitta and Eduardo Tapia*
Laboratory of Entomology and Biotechnology, Instituto de Investigaciones Agropecuarias—INIA La Platina, Santiago, Chile

*Address all correspondence to: etapia@inia.cl

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Chapter 2

Management of Pesticides from Vineyard to Wines: Focus on Wine Safety and Pesticides Removal by Emerging Technologies

Georgiana-Diana Dumitriu (Gabur), Carmen Teodosiu and Valeriu V. Cotea

Abstract

Grapevine (*Vitis vinifera* L.) represent an important crop, being cultivated in 2018 on 7.4 million hectares worldwide, and with a total production of 77.8 million tonnes. Grapes are susceptible to a large number of fungal pests and insects that may cause important economic losses, reduction of quality and undesired sensory characteristics in wines. A common practice in viticulture is the utilization of chemical reagents, as pesticides, that can insure constant production of high-quality grapes. The use of pesticides in vineyards is an old agricultural practice and although generally beneficial, some concerns are raising due to potential toxic compounds assimilation during wine consumption and human health risks. This chapter offers a complete overview of the most common pesticides used in vineyard and tracks them across grapes, winemaking stages and wines. The impacts of pesticide residues on phenolic compounds and volatile compounds are discussed in details, alongside with emerging technologies for removal of pesticide residues from grapes and wines.

Keywords: pesticides residues, winemaking stages, wine quality, pesticides removal technologies

1. Introduction

Grapevine (*Vitis vinifera L.*) represent an important economical and nutritional crop worldwide. Grapes can be consumed as fresh products or processed goods such as wine, jam, jelly, grape seed extract, vinegar, juice, raisins, grape seed oil and pekmez. Grape and wines are among the richest sources of phenolic compounds, including hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, flavan-3-ol monomers, flavonols, stilbenes, anthocyanins, oligomeric and polymeric procyanidins [1]. In their chemical composition we can find micronutrients, as vitamins B1, B6, C and minerals, as manganese and potassium.

Grapes are known to poses high amounts of carbohydrates and this makes them very vulnerable to damage by diverse fungal pests and insects [2]. High susceptibility to biotic stress of grape varieties can led to important economic loses, reduction

of wine quality and undesirable sensory characteristics. Vines and grapes can be affected by a large number of diseases, such as downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*), black rot (*Guignardia bidwellii*), Botrytis rot (*Botrytis cinerea*), Eutypa dieback (*Eutypa lata*), Phomopsis cane and leaf spot (*Phomopsis viticola*) and sour rot (*Aspergillus niger, Alternaria tenius, Botrytis cinerea, Cladosporium herbarum, Rhizopus arrihizus,* and *Penicillium spp.*), and many others. The high disease pressure and lack of genetically resistant cultivars have encouraged the use of large amounts of pesticides in vineyards, in order to generate stable yields and high-quality grapes [3]. During the grape production season and later on in winemaking, producers have identified small amounts of pesticides and named them residues. Every year, around 2 million tonnes of different pesticides are used worldwide and it is predicted that the use of pesticides in entire global production will increase up to 3.5 million tonnes [4]. Spraying grapes has to be done multiple times during the vine developmental stages and pesticide residues have been reported in literature by different authors [5].

The use of pesticides in vineyard is a conventional and ancient agricultural practice, which brings many benefits but, unfortunately, some disadvantages as well. Concerns regarding the exposition over a long period of time to pesticide residues present in wines have gained attention in the scientific community. In some cases, inappropriate agricultural practices are used during the application of these active substances in the vineyard. As a result, the amount of pesticide residues on grapes at harvest time exceeds the permitted level by national and international regulations. Alongside with the environmental risks, high amounts of pesticide residues may influence the quality of grapes and wines. Constant consumption of wine or grapes (and indirectly of pesticide residues), can provoke health issues to many consumers. Therefore, it is crucial to monitor the presence of pesticides and regulate their amount in grapes in order to prevent potential health risks. In the European Union, the maximum residue levels (MRLs) of pesticides permitted in products of vegetable origin intended for human consumption is establishes by Regulation 396/2005/EC [6]. Also, the MRLs limits and the analysis methods are regulated by various internationals directives [6, 7]. In grapes, the MRLs for pesticide residues often range between 0.01 mg/kg and 5 mg/kg depending on the pesticide, but in some cases higher limits are allowed.

Pesticide residues on grapes may be transferred during winemaking in the juice/must and later to the wine. This means a toxicological risk to consumers despite the fact that winemaking processes (crushing, pressing, fermentation, filtration and stabilization, etc.) can considerably decrease pesticides residues from wines [8]. Each phytosanitary product used in vineyards has a different mode of action which may explain the differences that were observed during analysis. Pesticide residues stability during fermentation and fining stages are factors of concern during winemaking. In red wine production, the maceration-fermentation stage take place in contact with grape skins, leading to greater residue amounts in raw wine. These types of residues can be adsorbed into solid state during fermentation or filtered out in the fining stages.

Grapes and wines are an indispensable part of people's lifestyle. The world surface devoted to the culture of grapevine is 7.3 million ha, and in Europe is 3.3 million ha [9]. Within the EU, according to the latest available data for 2020, Spain has the topmost area cultivated with vines (961 thousands of hectares-kha), followed by France (797 kha), Italy (719 kha), Portugal (194 kha), Romania (190 kha), Germany (103 kha). World wine consumption in 2020 was estimated at 260 million hectolitres (mhl) and in the EU at 165 mhl. Wine consumption was very high for USA-33.0 mhl, France-24.7 mhl, Germany-19.8 mhl, China-12.4 mhl, Spain-9.6 mhl, Portugal-4.6 mhl, Romania-3.8 mhl, Belgium-2.6 mhl and Switzerland-2.6 mhl [9].

The possible impact of pesticide residues on winemaking stages is a complex subject, and one that has a limited number of literature reports. The influence of pesticide residues on the grapes is a potential source of oenological concerns and can induce wine spoilage and undesired outcomes. The fermentation stage can be disturbed due to the active ingredients of pesticide residues in the must and thus, the quality and structure of wine can be negatively impacted. Pesticide residues can inhibit the yeast activity at the enzyme level and block the cellular metabolic processes of the yeast, leading to problems during the fermentation stage. Pesticide residues impacts on grapes can be influenced by the content of pesticides used in the vineyard, spraying method, spraying time, number of applications and the time difference between last application and harvest.

The morphology, size, and quality requirements of agricultural products are different, thus, influencing the overall content of pesticide residues. In winemaking stages, residues are transferred from the grapes to the wine, in accordance with the physical–chemical properties of their active ingredients, such as vapor pressure, solubility, boiling point, and octanol–water partition coefficient [10]. Processing of grapes using established winemaking techniques can influence the content of residues found in the juice and wine, but it is well established that, in general, wines have lower concentrations than must or grapes [11]. Environmental conditions such as sunlight, temperature and humidity can play a significant role in the kinetic and dynamic behavior of pesticides. In addition, other techniques for reducing pesticides are grape storage and washing processes that can minimize their potential adverse repercussion on human health.

A European Union recent report showed that pesticide residues could be found in more than 86% of grapes; moreover, multiple residues were reported in over 68% of tested samples (in total 2181 table grape samples) [12]. Under these conditions, it is highly recommended to speed up the pesticide residues analysis and come up with reliable, cheap and easy to use methods for identification, quantification and removal of such compounds from grapes, juices and wines.

2. Classification and toxicity of pesticides

Pesticides have a great variety of chemical structures, with diverse action mechanisms and applications. Nowadays, pesticides are presented in a large range of commercially products, with above 800 active components, belonging to more than 100 classes.

Pesticides can be classified bases on the pest type (A) and the origin (B) (**Figure 1**). In the first group of pesticides (A) are included: (1) herbicides, substances used to manage unwanted plant growth or to destroy weeds; (2) insecticides, used to kill infesting insects; (3) fungicides, used to control the propagation of fungi; (4) rodenticides that kill rodents; and (5) nematicides which kill nematodes or adversely affect nematodes. In the second group (B), pesticides can be categorized as chemical (synthetic) and biopesticides (biological or biorationals). The most outspread groups of pesticides are organochlorines, carbamates, pyrethroids and organophosphates. Organochlorines are the first important synthetic organic pesticides that belongs to the class of persistent organic pollutants (POPs). Biopesticides can be separated into two classes, that are, biochemical (hormones, enzymes, pheromones, natural insects, etc.) and microbial (viruses, bacteria, fungi, etc.).

Another classification of pesticides is based on the mode of action or mode of entry. Based on this, pesticides can be differentiated as non systemic, systemic, stomach poison, broad spectrum, disinfectant, nonselective, nerve poison, protectants and repellents. Moreover, pesticides can be classified using their acute toxicity.

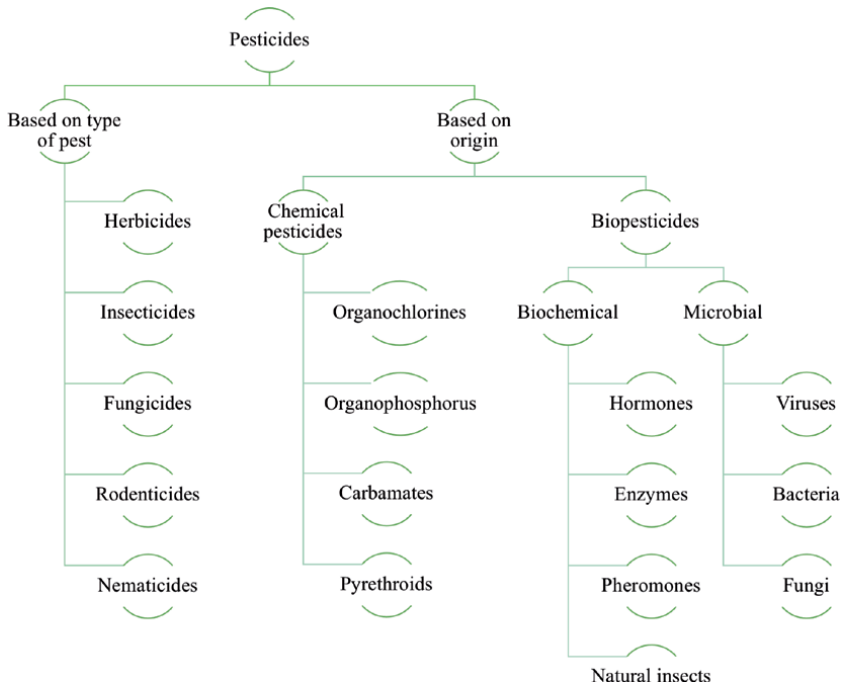

Figure 1.
Classification of pesticides.

WHO [13] grouped them in Class Ia = extremely hazardous, Class Ib = highly hazardous, Class II = moderately hazardous, Class III = Slightly hazardous, and Class U=Unlikely to present acute hazards.

Organochlorines (OCs) were among the frequently used pesticides in agriculture, and presented a high toxicity, with hazardous and bio-accumulation properties [14]. These types of pesticides are carcinogenic, persistent in the cycle of environmental degradation, belonging to group of chlorinated hydrocarbons. Moreover, they have high lipophilicity, low polarity and solubility in aqueous medium. OCs are forbidden and no longer used for agriculture in Europe, America and other countries. Organochlorines were substituted with other synthetic compounds such as carbamates, pyrethroids and organophosphorus. These synthetic compounds have a low price, low persistence in nature, high capacity to eliminate a vast number of pests.

The organophosphates and carbamates lead to disturbance in the normal functioning of the central nervous system (CNS), inhibiting the enzyme acetylcholinesterase (AChE) in (CNS) of humans and insects [15]. Organophosphates are widespread contaminants and are correlated with important toxicological threats to the soil, aquatic ecosystems and human health [16].

Pyrethroids are obtained from natural chrysanthemum ester containing natural chemicals, name as pyrethrins [17]. The synthetic pyrethroids have a longer environmental stability and half-life when as compared to the natural form. They have a particular insecticidal activity with reduced toxicity, operation by lagging the voltage gated sodium channel in the neuronal membrane.

Use of such pesticides in modern agriculture is regarded as beneficial for pest control, although residues accumulated in raw products or beverages are extremely dangerous to both human health and the environment. Consumption of wines that may contain residues of pesticides has a strong impact on human health, and may cause muscle weakness, respiratory disorder, paralysis, cancer, etc. [18, 19].

3. Management of pesticides from vineyard to wines

Grape growing and wine production are very complex processes, which start in the vineyard, continue in the winery and end in the consumer's glass. The environmental components, encompassing soil, topography, weather and climate have major impacts on vines growing and grape quality. Management practices in vineyards influence the accumulation of pesticide residues that can potentially affect the final wine chemical composition. Harvesting, transportation and transfer of grapes into the winery and later on the winemaking processes, can modify pesticide residues and gradually reduce or eliminate them.

Pesticide management techniques are constantly changing in accordance with the consumers and policy requirements. The promotion of sustainable viticulture and reduction of chemical inputs in vineyards arises new challenges and concerns for the entire viti-vinicultural sector.

Environmental conditions such as sunlight, temperature, soil, humidity and climate play a significant role in the kinetic and dynamic behavior of pesticides and grapes. Global warming is a key factor that provokes an increase in the accumulation of soluble solids in grapes, in combination with a lower amount of anthocyanins and acidity. As a cascading phenomenon, this slows, or even blocks fermentations and may lead to large economic losses in the winery. In addition, climate change presents a deep effect on the vine phenology, grape composition, winemaking stages, wine chemistry and microbiology and finally on the sensory attributes. Chemical composition of wines, aroma compounds, polyphenolic compounds, color, sensorial characteristics are all affected by the management of vineyards.

Management of vineyard is coordinated by humans and based on their decisions, many components may be affected. Grape quality is dependent on rows orientation, their training system, density, the calendar for pruning, trimming, fungicide treatments, or the way in which soil surface is managed, which comprise its tillage, the manipulation of the canopy structure and nitrogen fertilization [20]. High quality grape berries are influenced by the microclimate, sunlight and water levels. The light influences the evolution of grape volatile compounds, through the amount of light absorbed by the vine leaf area that determines the rate of photosynthesis. All these components generate an uneven distribution of favorable factors that may led to a high fluctuation of grape quality across different years.

Canopy management includes a series of common techniques, such as the plucking of leaves and head trimming. The first technique improves the microclimate of clusters, provides better fruit maturation, decreasing grapevine diseases incidence [21]. The second one, decrease transpiration and induces the lignification of the plant, balances the growth of branches and insulation within the foliage. Thus, wines resulted from defoliated grapes have higher fruity notes.

In order to obtain a high-quality wine, it is mandatory to have healthy grapes in the winemaking process. Vine growers have to be very careful in the prevention of parasite attacks in vineyards. Phytosanitary treatments used for common vine diseases such as botrytis, powdery mildew or downy mildew may provoke important problems during winemaking. Residues on grapes can be passed to the must and affect the selection and development of yeast strains [8]. Yeast can decrease the pesticides content in the wine. The persistence of pesticides depends on various factors such as the chemical characteristics of active ingredients, photodegradation, thermo-degradation and enzymatic degradation [22].

One of the essential pilons of the horticultural sciences for the control of insect-pests during the second half of XX century is Integrated Pest Management (IPM). There are various strategies to decrease the presence of pesticide residues in wine,

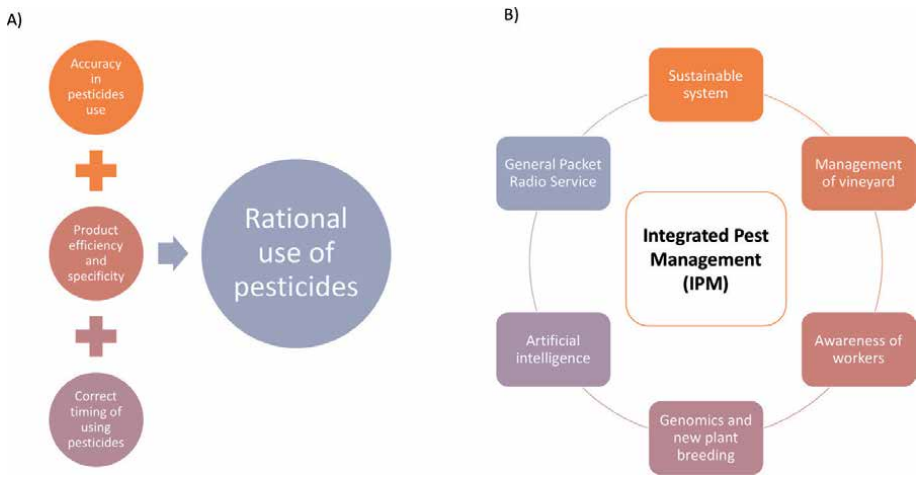

Figure 2.
Strategies used to remove pesticides in vineyards. A) Rational use of pesticides in the vineyards. B) Integrated pest management stategies.

such as treatments with sulfur, copper, or plant extracts as alternatives to synthetic products. Another strategy includes scheduled dosages and installation of a meteorological station to relay real-time weather data by General Packet Radio Service (GPRS) connection [23].

In the European Union [24] the use of copper fungicides in organic agriculture is restricted, being limited to 6 kg ha^{-1}per year [25]. Vallejo et al. [23] found that "weather station" was the most effective to decrease pesticide with wine-growing ecosystem.

IPM is considered as an environmentally friendly approach that can ensure sustainable production, constant yields and high-quality horticultural products [26, 27].

Sustainable agriculture is a key objective of the European Union and a focus of its sustainable development policies. Suitable remedial measures aim to decrease occurrence of pesticides toxicity and other health issues correlated with pesticides. Normally it employs mechanical, cultural and biological methods; allows use of chemical pesticides only when it is required; if possible, bio-pesticide usage, biocontrol and indigenous advanced [27]. Some strategies to reduce pesticide residues are presented below and in (**Figure 2A** and **B)**:

- Rational use of pesticides present advantages that include decreased expenses, decreased environmental impacts and increased safety (**Figure 2A**) [28].

- Organic strategy is used to increase organic cycles in horticulture, to preserve and improve extended soil fertility, to decrease all types of hazard provoked by pesticides extensive use.

- Awareness of workers: there is an urgent requirement to instruct the farmers and workers regarding the use of pesticides, their toxicity, and the risks of critical pesticide poisoning.

- Sustainable systems can decrease horticultural pesticide using the efficiency–substitution–redesign framework —precision and smart farming, substituting chemical inputs with biocontrol agents or mechanical weed control and improving the current cropping system.

- Genomics and new plant breeding techniques provide huge potential to increase the speed and technical opportunities in the development of resistant

cultivars; plant breeding is a long and complex process, which is often unable to keep pace with the rapid evolution of pathogens or the emergence of new pests — processes that are increasingly driven by globalization and climate change [29].

- Artificial intelligence in agriculture can help identification and classification of weeds, pests and diseases exactly and efficiently; photos taken by drones or from tractor-mounted spraying boots allow targeted spraying and decrease the overall applied pesticide quantities.

4. Effect of pesticides on wine quality

4.1 Pesticides effects on the polyphenolic content and antioxidant activity

A limited number of scientific reports could be found in the literature, regarding the influence of pesticides on the polyphenolic compounds in beverages. In the last years, studies on beer [30–32] and wine [33–35] chemical compositions have been published.

Dugo et al. [33] investigated the phenolic compounds of grapes and wines, after the use of pesticide treatments in the vineyard. Their results indicated that the antioxidant activity of wines was correlated to the content of phenolic compounds. In contrast, each individual phenolic compound was not homogeneous, and the contents were not correlated to various pesticide treatments.

Navarro et al. [30, 31] noticed on beers samples important differences in the total polyphenolic amount after fermentation for samples that contains residues of pesticides. Major reductions were recorded for propiconazole, 70.8%, myclobutanil, 43.0%, fenitrothion, 13.6%, and trifluralin, 6.8%, when compared to the control. Moreover, fenarinol, malathion, methidathion, nuarimol and pendimethalin were not influence by pesticide residues.

In 2011, Navarro et al. [32] observed that not significant differences on the total polyphenolic amount of beer after fermentation with fungicides. In contrast, statistical differences were noticed for the values of color intensity (lower) and tint (higher) in beer.

Recently, Briz-Cid et al. [34] reported that treatment with mepanipyrim decreased 1.2 times the level in monomeric anthocyanin, while polymeric forms increased 1.3 times. Also, after treatment with iprovalicarb the content in the monomeric anthocyanin increased by around 30%. Malvidin derivatives have been affected significantly, increasing up to 42%. Mulero et al. [35] noticed small changes of less than 10%. In his study, quinoxyfen and kresoxim-methyl have provoked the biggest increase in total anthocyanin, while the famoxadone, trifoxystrobin and fenhexamid reduced the anthocyanin content. No significant differences in antioxidant activity were observed. Similarly, Mulero et al. [35] reported that presence of pesticide residues did not influence the antioxidant activity in red wines.

In general, the treatment with fungicides did not change very much the concentrations of monomeric anthocyanins or flavan-3-ol monomers in wine [36]. Exceptions have been reported for treatments with boscalid + kresoxim-methyl which increased the amount of flavonoid groups with 58% and 36%, respectively. Mulero et al. [35] presented similar results for Monastrell wines from grapes treated with kresoxim-methyl. The treatment with quinoxyfen indicates an increase of phenolic compounds in wines when compared with control sample. In opposite, when trifloxystrobin was used it was observed a lower total content in phenolic compounds.

Castro-Sobrino et al. [37] indicated that the use of pesticides does not have an effect on anthocyanins. However, tetraconazole use led to a decrease of these compounds.

4.2 Pesticides effects on the aromatic profile

Wines represent a very complex matrix that contains hundreds of volatile aroma compounds. Aroma compounds originate from: i) varietal aroma that come from the vine and is released in the wine during the fermentation process. The most powerful varietal aromas are terpenoids, varietal thiols and methoxypyrazines; ii) fermentative aroma as a result of the synthesis of important volatile compounds through *Saccharomyces* and non-*Saccharomyces* yeast metabolism, are mainly constituted of volatile higher alcohols, acetate and ethyl esters, medium- and long- chain volatile acids, aldehydes, sulfur compounds [38]; iii) aging aroma either in bottles, in oak barrels or with oak chips, staves with the accumulation of characteristic new aroma compounds (**Table 1**).

Wine aroma can vary depending on the geographic area and terroir, viticultural practices, winemaking processes, type of aging and bottling. Moreover, other factors that have impact on the aroma compounds can interact with proteins, oxygen, polyphenols, polysaccharides, and thus modifying the sensorial characteristics of wines. A correct and controlled management of various methods or conditions of winemaking can help improve wine quality thorough removing the unwanted aroma compounds, the residues of pesticides or heavy metals, microbial contamination or oxidation, etc.

C6-alcohols belong to the group of C6-compounds and are formed during pre-fermentation stages, especially during harvesting, transport, crushing and pressing of grapes. These compounds are principally related to lipoxygenase activity in grapes or in must which produces aldehydes, then these, in turn, can be reduced to alcohols, by yeasts during fermentation stage. Higher alcohols are formed from their amino acid precursors, then are passed on to the wine, which are liable for fermentative aroma.

Reports suggested that the residual content of cyazofamid, famoxadone, mandipropamid and valifenalate was not affected by the synthesis of alcohols [47]. Similar results were published by other authors, regarding the chlorpyrifos, fenarimol, mancozeb, metalaxyl, penconazole, vinclozolin, fluquinconazole, kresoxim-methyl, quinoxyfen and trifloxystrobin in red wines [48] and with fludioxonil and pyrimethanil in white wines [49]. Interesting, opposite impacts were noticed for other pesticide categories. In red wines, a significant decrease of alcohols was observed when famoxadone, fenhexamid and tebuconazole were used [39, 48]. Contrasting, in white wines an increase of cis-3-hexen-1-ol content was observed in the presence of cyprodinil [49]. The same trend was noticed for tetraconazole in wines, in which the levels of cis-3-hexen-1-ol also increased with 55% [40].

A pesticides treatment that included fluxilazole showed that, in white wines, the content of isoamyl alcohols and 2-phenylethanol was increased with a direct correlation to the dose [50]. Moreover, other studies observed in white wines a decrease of 2-methyl-1- propanol and 3-methyl-1-propanol when fosetyl-A, mancozeb and iprovalicarb were used [41]. Results concerning the decrease of alcohols concentrations in the presence of some pesticides can be attributed to lower assimilation of the amino acid precursor by yeast or modifications in the biosynthesis of amino acids. However, a decrease in the quality of wine was noticed due to considerable increases in isoamyl alcohols contents [48, 49]. González-Álvarez et al. [47]

Pesticides	Pesticides losses	Quality and health risks of wine	Ref.
Iprovalicarb Mepanipyrim Tetraconazole	The fungicides mepanipyrim and tetraconazole exhibited a high dissipation rate during the winemaking process (93–98%); about 10–18% of iprovalicarb remained in wine.	The total content in the monomeric anthocyanin of iprovalicarb treatment increased by about 30%. Fungicides in wine do not only poses a health risk but also can alter fermentation and hence the quality of the wine	[34]
Metrafenone Boscalid + kresoxim-methyl Fenhexamid Mepanipyrim	no data	Presence of boscalid + kresoxim-methyl residues in must impairs the sensory quality of the resulting wine by diminishing its brightness and aroma. It increased the contents in monomeric anthocyanins (58%) and flavan-3-ols (36%), and also color lightness (20%), but decreased the contribution of the ripe (42%) and fresh fruits (59%) odorant series.	[35]
Fenhexamid Kresoxim-methyl Fluquinconazole Famoxadon Trifloxystrobin Quinoxyfen	no data	Wines from grapes treated with quinoxyfen shows an increase of phenolic compounds than the control. In contrast, the wine obtained from grapes treated with trifloxystrobin showed lower total concentration of phenolic compounds.	[36]
Mepanipyrim (Mep) Tetraconazole (Tetra)	no data	No effects on anthocyanins for mepanipyrim treatments were observed. A decrease of these pigments was registered when Tetra and Tetra-Form were applied; moreover Tetra-Form reduced phenolic compounds.	[37]
Tebuconazole	no data	The presence of residual levels of tebuconazole had no effect on varietal aroma compounds, terpene and higher-alcohol concentrations were essentially not changed; by contrast, C6-alcohol, ester and aldehyde concentrations differed significantly.	[39]
Mepanipyrim Tetraconazole	no data	Mep residues affected the release of varietal aroma compounds from their grape precursors, Tetra residues mainly affected the aroma biosynthesis pathways of the ethanol producing yeasts. Presence of Mep residues in grape must could contribute to wines having higher "floral" and "spicy" notes and lower "fruity" nuances while the presence of Tetra residues can contribute to wines having higher "floral and lactic" nuances.	[40]

Pesticides	Pesticides losses	Quality and health risks of wine	Ref.
Benalaxyl, Iprovalicarb, Pyraclostrobin	no data	Reduced the varietal aroma of wines attributed to geraniol. Increase in the fruity aroma due to several ethyl esters and acetates	[41]
Quinoxyfen	79–82% fungicide removal by alcoholic fermentation.	Quinoxyfen led to significantly lower ethylic ester levels. The addition of the fungicide did not seriously inhibit biomass production. A slight decrease of ethanol production in terms of both absolute value and conversion yield of ethanol produced per sugar consumed was, however, observed when the quinoxyfen concentration was increased.	[42]
Fenamidone, Pyraclostrobin, Trifloxystrobin	After winemaking, fenamidone, pyraclostrobin, and trifloxystrobin were not detected in the wine, but they were present in the cake and lees.	These three active ingredients could be used in a planning to obtain residue-free wines.	[43]
Iprovalicarb, Indoxacarb, Boscalid	Winemaking showed a complete transfer of all pesticide from grapes to the must, while in wine the residues were negligible due to the adsorbing effect of lees and pomace.	No risks of quality and safety defects.	[44]
Cyprodinil, Fludioxonil, Pyrimethanil, Quinoxyfen	Fludioxonil decreased most quickly during winemaking without maceration, whereas the decrease of pyrimethanil was the slowest in all cases. During carbonic maceration winemaking, the decay constant of cyprodinil was greater than that of the other pesticides.	The winemaker can also choose which winemaking process to follow depending on the residues.	[45]
Carbendazim, Chlorothalonil, Fenarimol, Metalaxyl, Procymidone, Triadimenol Carbaryl, Chlorpyrifos, Dicofol	After malolactic fermentation the concentrations of the active compounds chlorpyrifos (70%) and dicofol (30–40%) were the most significantly reduced.	In the case of dicofol, a substantial slowing of malolactic fermentation was observed when this compound was present at high concentration. Dicofol had a major inhibitory effect on the catabolism of malic acid (6–13% was metabolized), whereas chlorothalonil, chlorpyrifos, and fenarimol had only a minor effect (76–84% was metabolized).	[46]

Table 1.
Pesticides losses, quality and health risks of wine.

reported no significant differences in the alcohols level between control sample and wines treated with chlorpyrifos, cyazofamid, famoxadone, fenarimol, mancozeb, mandipropamid, metalaxyl, penconazole, valifenalate and vinclozolin.

The level of aldehydes increased slowly in the wine aging stage by effect of the oxidation of alcohols. The principal aldehydes that could be found in wines are benzaldehyde and phenylethanal [51]. Until now, results indicate that pesticides utilization do not influence the aldehyde contents [39]. However, in red wine, fenhexamid seems to be responsible for the increased content of benzaldehyde [48].

Sieiro-Sampedro et al. [40] founded that mepanipyrim influence the release of varietal aroma compounds while tetraconazole have a major impact on the aroma biosynthesis pathways of the ethanol producing yeasts. According to the OAV, the mepanipyrim could offer to wines higher spicy and floral nuances and lower fruity note whereas tetraconazole leads to higher floral and lactic notes. Mepanipyrim (Mepp) and Mep-Form generated a positive increase of the geraniol content, between 27 and 41%, benzyl alcohol between 91 and 177%, benzaldehyde between 51 and 111% and *trans*-isoeugenol between 37 and 308%. This trend was associated with the actions of yeast enzymes glycosidase and hydrolase of which activity is known to increase during fermentation.

Esters are produced by yeast during the alcoholic fermentation and play an important role in the fruitiness of wines.

The effect of cyprodinil, fludioxonil and pyrimethanil presented lower levels of hexanoate, ethyl octanoate and ethyl decanoate in white wines [49]. Also, grapes treated with quinoxyfen, kresomin-methyl and trifloxystrobin have decreased the content of ethyl dodecanoate and diethyl succinate in wines [41]. García et al. [49] observed an increased content of isoamyl acetate in the presence of cyprodinil, fludioxonil, chlorpyrifos, feranimol and vinclozolin. The level of ethyl acetate increased also when chlorpyrifos were used, whereas decreased its content with famoxadone and fenhexamid [48]. Other studies did not notice differences in ethyl ester and acetate levels in control sample and grapes treated with cyazofamid, famoxadone, mandipropamid and valifenalate [47]. Similarly, Noguerol-Pato [39] reported no significant variations, caused by treatments with tebuconazole, in the level of isopentyl acetate and most ethyl esters found in Mencía wines. On the other hand, residues of other pesticides seemed to increase the content of isopentyl acetate [41, 48].

Terpenes are found in grape skin, have an important role in varietal aroma and contribute considerably to the grape bouquet.

Oliva et al. [48] reported that treatment with some pesticides (famoxadone, fenhexamid, fluquinconazole, kresoxim-methyl, quinoxyfen and trifloxystrobin) presented an increase of terpenoic class in red wine comparative with control sample. Another study by González-Álvarez et al. [47] showed that cyazofamid and famoxadone treatments have a major impact in the synthesis of trans, trans-farnesol of white wines. Also, three fungicides (benalaxyl, iprovalicarb and pyraclostrobin) have altered the geraniol synthesis [41]. On the contrary, Noguerol-Pato et al. [39] observed that tebuconazole caused no important changes in the terpenoic content of red wines.

The treatment with famoxadone and cymoxanil led to a reduction in the content of isovaleric, caproic and caprylic acids, while valifenalate and cyazofamid increased the content of capric acid, according to González-Álvarez et al. [47]. In another study, the quinoxyfen, kresoxim-methyl, famoxadone, trifloxystrobin, fluquinconazole and fenhexamid content decreased the acid concentration in red wines compared with control sample [48].

Lactones are obtained through the intermolecular esterification of 4- hydroxy-acids. The use of pesticides on crushed Tempranillo and Graciano grapes did not affect the formation of lactones.

5. Emerging technologies to remove pesticides from grapes and wines

Pesticide residues in grapes and by-products can be a major concern to human health. The majority of grape products are consumed raw or slightly processed [52]. It is imperative to identify processes that are able to decrease and remove the pesticide residues from all horticultural products.

Certain processes, like washing [53], peeling [54], or cooking [55] have been reported in literature as good methods to decrease the content of pesticide residues and also reduce the risk of exposure to these phytosanitary products. However, some horticultural crops such as grapes are not subjected to a washing stage in their industrial processing line, and they are not peeled or cooked previous to consumption. Commonly, grapes are treated followed a phytosanitary scheme in the vineyard, harvested and then directly subjected to the winemaking process.

Proactive removal of pesticide residues from grapes and wines can be done by using decontamination techniques, classified as physical, physical–chemical and oenological methods (**Figure 3**). Apart from the classic methods used for reducing pesticide residues, the application of new or emergent technologies such as pulsed electric field (PEF) or ultrasounds, in the grapes and wines, is a current research hotspot.

5.1 Physical methods

Physical methods partially eliminate pesticide residues from grapes and wines are used on a small scale in the wine industry. Most of these techniques are not economically feasible for most small to medium size winemakers, even if nowadays, the modern beverage processing technologies aim at beverages safety and sustainable production.

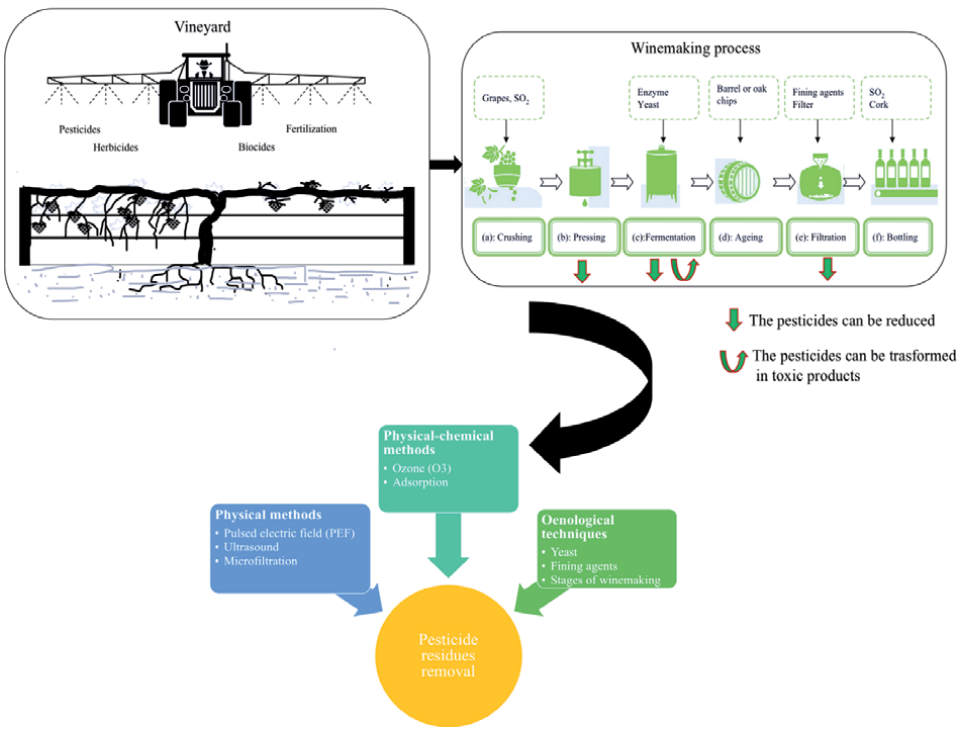

Figure 3.
Removal of pesticides from grapes and wines.

Pulsed electric field (PEF) method is an emergent non-thermal technology that induces a lower degradation of compositional and sensorial characteristics than the classical thermal processing. This method uses an electric field in the form of short or high voltage pulses. The beverage is placed into the electric field, between two electrodes for a short period, regularly in the microsecond scale [56].

Zhang et al. [57] reported that PEF method in apple juice can reduce the content of diazinon and dimethoate. The efficacity of PEF can be improved with increased process time and the strength of the electric field. Efficient removal of diazinon (47.6%) and dimethoate (34.7%) was realized when using 20 kV cm − 1 for 260 μs.

Delsart et al. [58] studied the impact of the same treatment on vinclozolin, pyrimethanil, procymidone, and cyprodinil in wine samples. Results revealed that PEF method can decrease the fungicide content and the major factors of influence were the electrical field strength and used energy level.

Ultrasounds represent a promising innovative and green method, which offers numerous advantages, such as simplicity, cheap, energy-saving. The principal limitations of this technique and its wide use in the industry can be solved by combining it with other compounds or treatments.

Ultrasonic dishwasher is a recent technique used in elimination pesticides from fruits and vegetables [59]. Ultrasonic waves provoke a phenomenon such as cavitations, which leads to the fast formation and violent collapse of micron-sized bubbles in a liquid medium. This method with tiny implosions that ensure the cleaning power, using the ultrasonic washing, was not exploited to its maximum potential. In a recent study, Zhou et al. [60] investigated the ultrasonic washing process to eliminate pesticides from grapes. Washing with the ultrasonic dishwasher proved to be more efficient for pesticides removal. Results showed residues decreased rates between 72.1% and 100% on grapes when comparing with normal water washing**.**

Another very promising emerging technology used for grape products is **microfiltration**. This method uses a membrane technology driven by pressure and, up to date has found many practical applications for pesticides reductions, offering several technological advantages [61]. Among the advantage of microfiltration are the high separation efficiency, low energy consumption, easy implementation and operation, absence of phase transition and non-use of additional solvents, which favor the solute recovery. Doulia et al. [62] investigated microfiltration in process of elimination of pesticides from a Greek wine, utilizing six membranes with the same pore size 0.45 μm. The membranes used were: cellulose acetate (CA), cellulose nitrate (CN), regenerated cellulose (RC), polyethersulfone (PESU), polyamide (PA) and nylon (NY). Results on the effectiveness of pesticides removal were as follows for white wine: cellulose acetate > cellulose nitrate > polyethersulfone > nylon > regenerated cellulose > polyamide and for red wine: cellulose acetate > cellulose nitrate > regenerated cellulose > polyethersulfone > polyamide > nylon. Another aspect found by the authors was that the bigger hydrophobicity and the lower hydrophilicity of pesticide, the higher the microfiltration effectiveness for both wines. Moreover, Doulia et al. [62] showed that the hydrophobic pesticide removal is more effective in red wines than in white wines, for all six membranes. This seems to be caused by the presence of higher amounts of hydrophobic polyphenolic compounds in red wine.

5.2 Physical: chemical methods

One of the known methods for pesticides removal is the chemical adsorption. This method is described as eco-friendly, low production of by-product waste and cost-effectiveness. Various types of adsorbents such as clay, activated carbon,

biochar and nanoparticles have been used for the adsorption of pesticides from grapes and wines. Adsorption techniques can be chemical, as bonding through ion-dipole interactions, weak Van Der Waals, forces, dipole–dipole, cation exchange and strong covalent bonding or physical adsorption [63]. Effective removal of pesticide residues depends on the pesticides concentrations, the wine fining agents, the type of compounds and the dosage.

Ozone (O_3) treatment is a new modern technique with various uses in food and beverage industry like as pesticide removal, water remediation and decontamination of fresh fruits. Ozone has been accepted by the World Health Organization (WHO), Food and Drug Administration (FDA) and by the Food and Agriculture Organization of the United Nations (FAO) for usage as an antimicrobial agent for the treatment, storage and processing of foods in gas and aqueous phases in 1997 [64]. Since that time the ozone treatment has been utilized in the agri-food-beverage sectors, in particular to control postharvest decay and extend shelf-life of fruits and vegetables [65]. It was shown that postharvest ozone treatments improve resveratrol and other phenolic compounds [66] and decrease pesticide residues [67].

Ozone can be used in various forms such as dry, watery and moist during the decontamination method. O3 in the beverage processes is used as an oxidant for pesticide content reduction. The percentage of pesticide removal depends on the ozone characteristics and not only on the chemical pesticides composition. Thus, it is obvious that specific conditions are necessary for the effectiveness of the ozonation process. The elimination of pesticides is influenced by different conditions of application (pH, temperature and humidity), organic matter content, ozone concentration, production rate and form of application (aqueous and gaseous) [68].

The principle of this technique consists of ozone generation by the passage of air, or oxygen gas through a high-voltage electrical discharge or by ultraviolet light irradiation [69]. The product of ozone degradation is oxygen; thus, it leaves no residues on treated items. There are other possible benefits of ozone, like the elimination of mycotoxins [65], pesticide residues and microbiological control of food products [70].

In 2015, Dordevic and Durovic-Pejcev [71] affirmed that juice processing may eliminate the pesticide amounts by using washing/cleaning, pulp-removing, pressing, squeezing, clarification (like centrifugation, enzymatic treatment and filtering) and heat treatment (like boiling, pasteurization and sterilization). Botondi et al. [72] suggested to utilize ozone fumigation postharvest, in order to analyze microorganisms and evaluate the influence on polyphenols, anthocyanins and cell wall enzymes during the grape dehydration for wine production. Ozone treatments decreased yeasts and fungi by 50%. Moreover, a treatment that used shock ozone fumigation before dehydration decreased the microbial count during dehydration without influencing the polyphenol and carotenoid amounts. In 2018, Karaca [73] studied the removal of pesticides from grapes by exposing fruits in ozone-enriched air. Gaseous ozone rich atmosphere led to a 2.8-fold higher removal of azoxystrobin fungicide than control sample. Both phases, gaseous and aqueous ozone techniques displayed 67.4% and 78.9% decrease of chlorothalonil residues from table grapes [74]. The differences in the efficacity of pesticide residues may be assigned to the diversity in the structure of the pesticides.

Activated carbon (AC), is generally used in winemaking to remove phenolic compounds, pigments and off-flavors. AC has high and broad affinities especially for benzoid and non-polar substances. Activated carbon shows large positive effects on reduction of pesticides, due to its high adsorption capacity, large surface area and high porosity.

Sen et al. [75] studied the influences of activated carbon with low, middle, high doses on the removal of vinclozolin, penconazole, endosulfan, imazalil, nuarimol

and tetradifon used in viticulture. The amount of imazalil decreased in white wine with middle and high doses of activated carbon, but low dose of activated carbon removed 92.96% of imazalil. This result can be associated to the high adsorption surface of carbon and to the limited interference from the wine chemical compounds.

Nicolini et al. [76] investigated whether small amount of pesticide residues can be removed adding a low dose of activated carbon during fermentation. AC decreased up to 130 μg/L of fungicides in the white wine samples studied. Results obtained in wines fermented with activated carbon had 30–80% lower fungicides as compared to the control. An exception was found in the case of iprovalicarb which did not significantly decreased.

Bentonite is a natural montmorillonite clay and in nature has Mg^{++}, Ca^{++}, Na^{+}, aluminum and silicon oxide forms. The most used form of bentonite in winemaking is sodium bentonite, which has a large adsorption surface. This surface has a strong negative charge, and it allows ion exchanges and other electrostatic interactions. Bentonite sodium is used largely in winemaking for the elimination of positively charged proteins. Among the disadvantages of bentonite are the nonselective elimination process and the reduction of valuable aroma compounds from wines [77, 78].

Sen et al. [75] reported that bentonite had a major effect on decreasing the concentrations of imazalil (96–98%), endosulfan (81–87%), and penconazole (84–95%). However, bentonite influence on nuarimol and tetradifon was limited, removing between 15 and 33% and 25–39%, respectively. Bentonite had no influence on the elimination of vinclozolin. Ruediger et al. [79] has shown that 500 and 2500 mg/l of bentonite eliminated a large amount of pesticides from white wines. The authors have found that there was not a clear effect of an increased dose of bentonite on triadimenol and metalaxyl.

Navarro et al. [80] showed that filtration of wines, previously clarified with bentonite and gelatin, lead to the removal of 2% metalaxyl, 7% fenarimol, 25% penconazole and 28% vinclozolin. During maceration stage, the rate remaining of chlorpyrifos, penconazole and metalaxyl was 90%, while the percentage of fenarimol, vinclozolin and mancozeb was lower (74–67%).

Likas et al. [81] reported that processing of treated grapes into wine almost removed residues for flufenoxuron and lufenuron resulting in residue-free wine, whereas tebufenozide was found in wine at concentrations from 0.13 to 0.26 mg/L. Among the fining agents used, bentonite, potassium caseinate, gelatine–silicon dioxide and polyvinylpolypyrrolidone did not actually eliminate residues from wine, while charcoal very effectively removed tebufenozide residues. The pesticide residues in grapes presented a low removal for 42 days after phytosanitary treatment, with dissipation rates varying from 0.011 to 0.018 mg/kg day. The pesticide residues have shown for 0.27 mg/kg for flufenoxuron, lufenuron and 0.68 mg/kg for tebufenozide, and their concentrations were lower than the maximum residue limits (MRLs).

Chitosan is a biopolymer obtained from chitin and comprises N-acetylglucosamine and glucosamine units. These properties of the chitosan structure give its flexibility and heterogeneity. Hydrophilic functional groups cannot alter chitosan's hydrophobic nature and support adsorption [82].

Venkatachalapathy et al. [83] studied the pesticide removal efficacy, when using chitosan fining agent in grape juice during the clarification stage. In this study, pesticide removal efficiency of chitosan ranged from 54–72% at 0.05% chitosan concentration, and increased up to 86–98%, when higher chitosan concentration was used (up to 0.5%). Results showed that 0.05% chitosan had the highest pesticide removal efficiency (72%), when compared other clarifiers. Also, investigations showed that

the optimal pesticide elimination was achieved using chlorpyrifos (98%) and ethion (97%) at chitosan for 1 h incubation continued by phorate (96%), fenthion (95%), fenitrothion (94%) and diazinon (86%) at chitosan for 2 h incubation time.

In recent years, a new carbon rich adsorbent (38–80%), **biochar**, attracted remarkable attention. Biochar is produced by thermal conversion under oxygen free environment [84]. Yuan et al. [84] expressed that the biochar surface brings negative charges because of the occurrence of organic groups. Biochar can be used for the elimination of different toxic compounds such as pesticides, heavy metals, antibiotics and dyes. Biochar has unique characteristics such as higher pore volume, larger surface area, high environmental stability, low cost and extensive raw material sources [85]. Moreover, other materials like clay, zeolite, mesoporous materials were also used for the removal of pesticides from grapes and wines.

Grape pomace (GP) is a by-product of various grape based manufacturing processes, such as juice, jam-making, wines, etc. The GP biomass represents around 20–30% of the residual biomass of grapes. European countries reported GP wastes of about 1,200 tons per year. Yoon et al. [86] investigates in his work the adsorptive comportment and mechanisms of grape pomace-derived biochar (GP-BC). Pesticide cymoxanil removal rates were assessed during this study. Biochar produced at 350°C achieved the maximum adsorption capacity of 161 mg CM/g BC at pH 7 for cymoxanil. Thus, cymoxanil adsorption was attributed to the combined influences of metal and hydrophilic interaction.

Angioni et al. [44] has researched the transfer from grapes to wines during the entire winemaking process for some pesticides. The concentrations found in grapes were under limits set by the EU, having the amounts 0.81, 0.43, and 4.23 mg/kg for iprovalicarb, indoxacarb, and boscalid, respectively. The obtained results showed that all pesticides have been transferred from grapes to the must, whereas in wines the residues were insignificant. For pesticides, the clarification stage presented a good elimination of these toxic compounds from wines.

5.3 Oenological techniques

Winemaking processes have the potential to remove, degrade or decrease pesticides content in grapes. This is achieved mainly through stages of winemaking, such as pressing, filtration, adsorption or through microbial processes occurring during the fermentation stage [87, 88].

In the first stages of winemaking, in pressing and maceration process, the pesticide residues on grapes are decreased notably. Thus, a considerable amount of toxic compounds remain in the cake and lees, and a small quantity migrates into the must [89]. In the next stage, in alcoholic and malolactic fermentation, yeasts destroy some part of pesticide residues. Another important stage in which takes place the reduction of pesticide residues is the clarification step [90].

Pan et al. [91] found that the whole process can reduce the zoxamide residue in red and white wines. Peeling process has an important influence on the decrease of zoxamide, because a high content of this pesticide was retained by the grape skin. These results can provide more accurate risk assessments of zoxamide during winemaking process. Pazzirota et al. [92] found that pesticide distributions over the different stages of winemaking process were clearly dependent on the affinities of pesticides to organic or aqueous fractions in the process. The pesticide contents decreased from grape to wine. Decreases from fermentation stage during maceration are due to pesticide affinities for solid residues present in the sample for cyprodinil and imazalil.

Yeast have the ability to decrease pesticide residues from wines, by degradation and/or adsorption. The removal of pesticides during winemaking has been widely studied [93]. In this process, the main agent for adsorption is the yeast cell wall,

containing polysaccharides as basic building blocks. It has been shown that the principal fraction of mannoproteins is released in the first week after the alcoholic fermentation has finished. In this stage the dominant adsorptive action is noticed. Also, at the end of the alcoholic fermentation, *bâtonnage* is used to obtain higher quality wines. The mannoproteins are released and the adsorption of pesticides take place [94]. However, not only strain properties, but also differences in the binding affinity of pesticides, are important factors. The adsorption of yeast lees is different among strains, and due to the cell wall structure, physicochemical conditions, especially pH, influence the adsorption ratio [94].

Elimination of pesticides by degradation is an uncommon process. Yeast have the ability to degrade some pesticides from the pyrethoid class and insecticides thiophosphates class [95]. During fermentation, yeasts partially degraded quinoxyfen and adsorbed it completely [89]. It is been shown by Cabras et al. [89] that fenhexamid did not affect alcoholic fermentation, whereas a great content of pyrimethanil (10 mg/L) was found to significantly diminish the anaerobic growth of *Hanseniaspora uvarum* [96]. In other studies, the presence of pesticides has been found to stimulate yeasts, especially *Kloeckera apiculata*, which produced more alcohol [97]. Oliva et al. [98] found that no fungicides delays or inhibits fermentation processes. Also, the evolution of yeast populations during fermentation follows the normal multiplication processes of the species.

6. Conclusions

Increased population, higher demand from quality beverages, rapid climatic changes and the need for more phytosanitary treatments constitute to a wine industry that has to focus more on sustainable practices, high grape yields and minimized health risks. Conservator winemakers that use adequate agricultural practices can limit potential negative effects that are linked to higher pesticide concentration in wines. However, the high pressure of climatic conditions, increased pathogen virulence and mutations into new variants can increase the quantities of pesticides needed in vineyards and led to potential human health risks. Large pesticide quantities may affect negatively the water and soil quality, leading to undesired effects on the animals, plants and human communities.

Different techniques have been used successfully to remove pesticide residues form grapes and wines. Technologies such as pulsed electric field (PEF), ultrasounds (US), microfiltration, ozone (O_3), adsorbents used during pressing, fermentation and filtration are nowadays implemented by many winemakers. However, preventive methods applied directly from vineyards and emergent technologies should be utilized to produce grapes with tiny amounts of pesticides. Effective pesticide management requires actions supported by a very clear and transparent legal system and toxicity regulations.

Integrated pest management strategies could provide a more efficient control of pesticides use and limit the residues. Utilization of precision spraying and local treatments can reduce the pesticide residues negative impact on the environment and potential human health risks.

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Author details

Georgiana-Diana Dumitriu (Gabur)[1], Carmen Teodosiu[1*] and Valeriu V. Cotea[2]

1 "Gheorghe Asachi" Technical University of Iasi, Iasi, Romania

2 "Ion Ionescu de la Brad" University of Life Sciences of Iasi, Romania

*Address all correspondence to: cteo@tuiasi.ro

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Chapter 3

Alternatives to CU Applications in Viticulture: How R&D Projects Can Provide Applied Solutions, Helping to Establish Legislation Limits

Mario De La Fuente, David Fernández-Calviño, Bartosz Tylkowski, Josep M. Montornes, Magdalena Olkiewicz, Ruth Pereira, Anabela Cachada, Tito Caffi, Giorgia Fedele and Felicidad De Herralde

Abstract

Copper (Cu) and its based preparations have been used for over 200 years to control fungi and bacterial diseases in cultivated plants. Downy mildew caused by the obligate biotrophic oomycete *Plasmopara viticola* is one of the most relevant and recurrent diseases of grapevines. Recently, the use of Cu is being limited by some regulations because of its high impact at different levels (health and environmental problems). Due to its accumulation in soil, this metal causes a little controversy with the principles of sustainable production. Therefore, international legislation and initiatives have recently been arisen to start limiting its use, with the main goal to replace it. In this framework, some alternatives have been tested and others are recently being developed to replace, at least partially, the use of Cu in viticulture. Many of them, are being developed and tested under the scope of research and development EU funded projects. To not compromise sustainability targets in viticulture, results from these R&D projects need to be considered to assess the present risks of using Cu in viticulture and to better support establishing limits for its applications, considering soils vulnerability, while no sustainable alternatives are available in the market.

Keywords: *Plamospara viticola*, Sustainability, Copper, Downey mildew, innovation

1. Introduction

Cu based preparations have been used for over 200 years to control fungi and bacterial diseases in cultivated plants. Downy mildew caused by *Plasmopara viticola*, which occurs throughout the world, is one of the most destructive of all grapevine diseases. Cu-based fungicides are used to control grapevine diseases even in organic vineyards. Their use had a worldwide development after the accidental

discovery of a Bordeaux mixture in the 1880s', when the winegrowers of this region, using a mixture of Cu, sulphate and lime to avoid people to pick up and eating these grapes. Due to this practice, a French scientist called Millardet noted these covered grapes did not present a downy mildew damage. By 1885, Millardet completed experiments, that confirmed the capability of this mixture to control this disease at a relatively low cost. Therefore, the Bordeaux mixture became the first fungicide to be used on a large scale, worldwide level [1].

Cu is an essential element for plant growth occurring naturally in soils in concentrations between 5 and 30 mg kg^{-1}, although exceptionally in soils developed on some type of basic parent material may reach values between 100 and 250 mg kg^{-1} [2, 3]. However, the historical use of Cu based-fungicides in vineyards leads to important increases of Cu concentrations in soils, because due to its low mobility it tends to accumulate in the upper soil layers, after rainfall removal from the vines, deposition of the senescent leaves or accidental spills [4]. Thus, in vineyard's soils in Europe is possible to find Cu concentrations higher than 100 or even 200 mg kg^{-1}, while in subtropical areas of Brazil values higher than 1000 mg kg^{-1} were already found [5].

In 2018, a new publication of JRC [6] that maps Cu concentration in European Union topsoils, finds that vineyards have almost three times the average soil Cu concentration (49.26 mg/kg compared to the overall average of 16.85 mg/kg), followed by olive groves (33.49 mg/kg) and orchards (27.32 mg/kg). However, Cu distribution in the soil is strongly influenced by climate and topsoil properties. The climate will affect the number of treatments and leaching of Cu into soils, whereas soil properties have a strong influence on its behavior in this matrix [3, 4]. Once in soils, Cu is strongly complexed or sorbed by OM, oxides of Fe and Mn and clay minerals, whereas low pH values tend to promote its mobilization [3, 5].

The continuous increase of Cu concentrations in soils devoted to vineyards cause an increasing concern because high concentrations of Cu in soils may cause negative impacts on soils-organism functions and diversity, and also on vineyards surrounding ecosystems. Indeed, environmental values of Cu commonly found in soils under inputs of Cu-based fungicides are shown to be toxic not only to non-target soil organisms like worms and microbial communities but also to aquatic organisms such as *Vibrio fischeri* and *Daphnia magna* [3]. Values ranging between 26.3 and 31.8 mg·Cu $kg^{-1}$of soil, which are lower than for example the mean Cu concentration found in European vineyard soils, has been proposed to guarantee the protection of terrestrial elements and ecosystems functioning [7]. Nevertheless, when assessing the toxicity of Cu and its impacts on the environment, not only total concentrations in soils should be considered, but also its bioavailability and mobility, which are both strongly affected by the soil properties and aging processes [3]. The toxicity of Cu is also dependent on the chemical species present in soil solution (i.e. free and complexed) [3, 5]. The mobility of Cu influences its ability to migrate through the soil profile up to other environmental compartments, for example, reaching water masses more easily [3].

Due to the environmental problems related to the accumulation of Cu in soils and potential contamination of the aquatic environment, since 2007[1], Cu use has been limited by European regulation, being a little controversial with principles of organic farming. Furthermore, the EU regulate by laws[2] the list of approved active substances and its potential risks for protection of water and non-target organisms concerning countries to realize e.g. buffer zones to these identified risks

[1] Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and labelling of organic products.

[2] REGULATION (EU) No 540/2011. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R0540&from=EN.

and risk mitigation measures where appropriate. In the past, regular inputs of Cu up to 30 kg·ha^{-1} (per every 5 years) were frequently attained and allowed. After each application, the residue is typically accumulated in the upper 15 cm of soil, given the high affinity of Cu with the soil organic matter (SOM), that contains several reactive groups, like carboxylic and phenolic groups, which can complex Cu cations, after deprotonation, reducing its mobility in soils [8].

Not only in Europe, in California, but there were also some studies which have shown that there was an increase in the use of Cu in vineyards, caused an accumulation in soils from 6 to 9 kg·ha^{-1} [9] during the last years of the 90s'.

Nowadays, and after recognizing the risks of copper accumulation in soils, the use of Cu in the European vineyard is limited to a maximum of 28 kg Cu·ha^{-1} and over 7 years[3]. This limit is usually applied to organic farming, whilst for conventional viticulture, there are alternative plant protection products available resulting in much lower Cu quantities. Some countries e.g. Germany and Austria had more strict limits (3 kg·yr.$^{-1}$·ha^{-1}) when necessary. Private organic organizations, like. Biodynamic growers with Demeter certification[4] and other biodynamic groups as ECOVIN, Bioland, Natruland, Bio-Austria, etc. can only use a maximum of 3 kg·yr.$^{-1}$·ha^{-1}. In France, the national legally allowed application rate of Cu is 6 kg·yr.$^{-1}$·ha^{-1} with flexible mechanisms (30 kg·yr.$^{-1}$·ha^{-1}) for organic agriculture. Furthermore, the France Minister of Agriculture and Food launched a national program "Ecophyto[5]" aimed at reducing the use of pesticides in agriculture.

Other standards like Slovenian or the Australian and New Zealand guidelines, focus on risk assessment of contaminated sites and give support decisions about remediation measures. In general, where total Cu concentrations in soil exceeding 60 mg·kg^{-1}, sites require environmental investigations [10, 11].

Despite the efforts for reducing the use of copper, the situation is challenging for organic agriculture for which synthetic active substances cannot be part of the solution.

2. Possible different alternatives and approaches to the use of Cu

2.1 Animal origins

Chitoplant©, Enzicur© and other extracts from animal origin (*Lumbricus humus*, propolis, milk protein and hydrolyzed proteins) have been proposed to reduce downy mildew symptoms [12], as they can form semipermeable films protecting plant tissues and stimulating plant's defense mechanisms.

Chitosan hydrochloride is a kind of resistance promoter that enhances plant protection against pathogenic infections. It has proven effects against bacteria and fungi (such as *P. viticola*), and it was approved for use in agriculture as a plant protection product by European Commission[6] [13].

However, their impacts on grapes and must quality have to be carefully assessed, as some studies point to negative effects. Garde-Cérdan et al. [14]. observed that both copper hydroxide and chitosan applications to the grapevines decreased the

[3] REGULATION (EU) 2018/1981 of 13 December 2018: total application of maximum 28 kg of Cu per hectare over a period of 7 years; Member States may in particular decide to set a maximum annual application rate not exceeding 4 kg/ha of Cu; expiration of approval: 31 December, 2025.

[4] https://www.demeter.net/wp-content/uploads/2021/04/20201204_bfdi_standard_for2021_final_sc.pdf

[5] https://agriculture.gouv.fr/le-plan-ecophyto-quest-ce-que-cest

[6] Regulation (EU) number 563/2014 of 23 May 2014, following Regulation (EC) number 1107/2009 of the European Parliament and Council. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32014R0563&from=EN

concentrations of all amino acids in must, except for Lys, and only when chitosan is applied alone. Romanazzi et al., [13], also recorded lower net photosynthesis, stomatal conductance, leaf area, and weight of leaves and pruned branches, as a consequence of chitosan treatments. The authors concluded that these side effects may be very risky for obtaining high berry quality.

Lactoperoxidase system (Enzicur©) is a natural anti-microbial system usually employed in the control of powdery mildew in various crops[7]. The product is based on naturally occurring salts (potassium iodide and potassium thiocyanate) and the lactoperoxidase system, active in different animals including in the bovine liver. Enzymes (lactoperoxidase) and substrates. The LP-system is a non-immune defense system, that promotes the formation of reactive oxygen species that inactivate microorganisms by protein's peroxidation.

2.2 Biocontrol agents (BCAs)

Bacillus subtilis (Serenade Max©) and *Trichoderma harzianum* (Trichodex©) has been found as promising candidates for replacing Cu as a biocontrol agent for protecting against downy mildew [12], and other fungi diseases.

Among some tested antagonists, the highest efficiency was observed for *Trichoderma harzianum*-based products. Its efficiency was significantly higher in the treated plot when compared with untreated one but decreased just before harvest. However, this *Trichoderma harzianum*-based product did not provide a level of *P. viticola* control similar to Cu in some trials [15]. Despite the positive results found in some experimental studies, it was realized that the ability of *Thricoderma* (T39) to induce resistance depends on grapevine cultivars. Thus, it is necessary to understand which are the molecular components and signaling pathways modulating the response to this resistance inducer to apply this biocontrol to the most responsive cultivars, enhancing the benefits of this biocontrol treatment [16].

Other results [17] showed the relevance of environmental conditions on BCAs activity (four-year trial). Prevention of fungal sporous germination at least in some years could means an interaction between the pathogen and the microorganism that can lead to a reduction of severity of primary foci.

On other hand, *Bacillus* and *Trichoderma* strains have a great ability to produce a wide range of active molecules with broad effects on the control of different grapevine diseases, by preventively inducing plants systemic resistance or inhibiting other fungi diseases development.

These works show that microorganisms could be a promising tool to reach a reduction of primary inoculum and thus contribute to a low impact and sustainable agriculture.

2.3 Cultural practices

In the case of an epidemic disease like the downy mildew, combat strategies relied only on chemical control and its optimization. Sanitation measures targeting to reduce the overwintering inoculum and therefore, to reduce early and linearly the primary infection, and regulation of the crop load are a good management strategy [18].

Another relevant and additional strategy is the strict regulation of Cu spray rates. In the field, rates between 200 and 400 g Cu·ha^{-1} (equivalent to 5 and 10 mg Cu·m-^{2}, respectively) was able to significantly reduce downy mildew (72–89% efficacy). These confirmed results (previously obtained from leaf disks assays in the lab), provided sufficient control, although it depends on the infection pressure [19].

[7] https://www.koppert.mx/enzicur/

Forecasting models linked to Cu applications could be an interesting approach. **Coptimizer**[8] is a model-driven decision support system designed to help growers to optimize and track the use of Cu-based fungicides against grapevine downy mildew in European organic viticulture. Results showed that by using Coptimizer (including historical data and several experiments under field conditions), growers could be able to maintain the same level of protection applying only half the amount of the fungicide [20].

An innovative cultural practice has been recently tested consisting in the application of different cover crops mixtures to interfere with the dispersal of the soil-transient pathogen, such as *P. viticola* [21]. Fall sowing of cover crops allowed to have enough vegetation in spring, during the most relevant period of downy mildew primary infections, to delay the onset of first disease symptoms and reduce the final incidence of the epidemic. This cultural practice can result in a final saving in treatment numbers as well as a reduced amount of copper used during the first seasonal treatments.

In summary, when *P, viticola* pressure is low to intermediate, a reduction in the sprayed Cu quantity provides the same efficiency as standard strategies and allows to decrease two-fold to three-fold the sprayed Cu quantity [15].

2.4 Inorganic materials

Some inorganic salts have shown promising results under controlled conditions (greenhouse and potted plants) like potassium bicarbonates (Armicarb© and SaluKarb©); K-P product based on betaine, carbohydrates and amino acids (Gro-stim©); N-K products with oligosaccharide and glutathione (Kendal©) or Aluminum oxide and silicon oxide with S (Ulmasud©) showed to be as effective as Cu hydroxide treatment. However, in field trials, only the potassium bicarbonate (Armicarb©) provided control of infection on bunches greater than 60% [17].

2.5 Microbial and plant product extracts or derivates

Under controlled conditions (greenhouse and potted plants), some microbial extracts have shown a good efficacy to control downy mildew [17]. Extracts from inactivated *Pseudomonas aureofaciens* (Agat 25 K© and Diamant©) were an effective treatments at concentrations above 10%. This product was effective in field trials, providing control of infection on bunches greater than 60%.

Many plants' oils or water and alcohol extracts showed reduce downy mildew expression compared with the untreated control [12, 17], under controlled conditions (greenhouse and potted plants):

- Siva 50©, and Tecnobiol© (fatty acid-based products like gibberellic acid-GBA plant wash soap), significantly reduced downy mildew expression.

- Penergetic-p liquid© (cane sugar) and Phyto-Vital© (lignin derivate) were the only natural derivative treatments that showed the same effectiveness as Cu hydroxide.

Therefore, plant and other extract products isolated used without Cu can reduce their efficacy when *P. viticola* cause a high pressure in the vineyards.

Hedera helix (leaves in water), *Quercus spec*. (bark in alcohol), *Primula veris* (roots in water), *Rhamnus frangula* (roots in alcohol), *Solidago spec*. (leaves in

[8] https://www.haifaup.co.il/startup/coptimizer

alcohol), *Salix spec*. (bark in water) showed promising effects in the laboratory [22], and these effects increased with the concentration of plant material used to obtain the extract. Extracts from *Rhamnus* and *Primula* had significant effects, reducing disease severity by 30–35% if applied after infection.

In field trials, some of the extracts, such as those from *Chenopodium quinoa*; *Inula viscosa*; *Melaleuca alternifolia* (Timorex©); *Salix alba*; *Solidago virgaurea and Salvia officinalis* provided more than 60% of control of bunches infection [17].

In general, preventive effects were much better in lab conditions (70–90% reduction of disease severity) than the results in field experiments (34–40% disease reduction) for the species tested [22]. In particular, *Yucca schidigera* (Norponin BS© liquid and Saponin©) has been also found as some of the most promising candidates for replacing Cu, because it provided more than 60% control of leaves and bunches infection [17]. However, some variability in *Yucca* extract efficiency under a low *P. viticola* pressure was already observed in some studies [15].

Trials with potted plants showed that *Salix* extract is a promising alternative to Cu, with no risk for the development of *P. viticola* resistant strains. Salix extract was as efficient, being the 4th day between elicitation and inoculation the appropriate moment to control the disease. Nevertheless, its action is strictly preventive and *Salix* extract should be applied before rainfall splash dispersion of fungi, which are impossible to forecast and in case of strong pressure this protection could be insufficient [23].

Therefore, available results also showed that the use of plant extracts (alone or in combinations among them) can reduce the doses of Cu and should be tested in future as a real alternative.

2.6 Synthetic materials

Under high *P. viticola* pressure, Cu-based treatments and potassium phosphonate (PP) are the most efficient products to control downy mildew. Beta-aminobutyric acid (BABA), benzothiadiazole, and high levels of polyoxyethylene sorbitan monooleate (Tween 80©) were as effective as the Cu hydroxide treatments in indoor trials [17], but no relevant effects were recorded in field trials.

Clay-based treatments such as Mycosin© are promising alternatives, giving in some trials a level of protection higher than 60% in leaves and bunches [15], but it is important to understand the impact of Al cations provided by this product. However, under a high disease pressure, the efficiency of these clay-based products is low for commercial vineyard protection.

Some vineyards trials in Germany and Austria showed that PP has a direct effect on *P. viticola*, and in addition, it activates the plant's defense mechanism (EFSA 2012[9]) which is one of the basic principles of organic plant protection, as stated in the European Organic Regulation[10]. PP is absorbed by the plant and systemically distributed. Due to the distribution through the plant and the resistance-inducing effect, this substance particularly protects newly grown leaves and shoots. It also reaches the pathogens that have already penetrated the leaves. Apart from the protective effects, the substance also has a curative effect during the first days of infection and incubation (approx. 25% of elapsed incubation time).

PP was used in organic viticulture in a few countries as a plant strengthener until 2014. When used until the end of the flowering period, it showed great support of Cu products in protection against *P. viticola* under high infection pressure.

[9] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2012.2963

[10] EU No. 834/2007

Efficacy of PP, stone meal as well as new Cu formulations, has been recorded as good reference treatments (Folpan 80 WDG©, a.i. folpet© and "organic standard" mixture of Cu, sulfur and stone meal), when the *P. viticola*. The pressure was low, considering the low amount of total Cu applied (less than 2 kg/ha), the results were promising [24]. Moreover, the use of PP as a plant protection product in organic vineyards contributes to a Cu use reduction to levels <3 kg of pure $Cu{\cdot}ha^{-1}{\cdot}yr.^{-1}$, and it has been a practice adopted in Germany and Austria. Therefore, PP can be considered in Cu-reducing strategies.

However, PP were registered as plant protection agents in the EU and therefore, not listed or allowed to use in organic viticulture. This led to big problems in years with high infection pressure in different regions all over Europe (like in 2016).

2.7 Other or new Cu formulations

New Cu formulations available in the market showed efficacy similar to Cu hydroxide, however, are not efficient at low concentrations. Cu is a preventive fungicide allowed in organic agriculture that is active only in tissues where is applied (i.e. it is a non-systemic substance), so plant growth results in unprotected tissues. In areas where disease incidence is high, weekly Cu applications are made by growers increasing the risk of exceeding the fixed threshold.

Some low Cu formulations were able to control grape downy mildew in the field using a third (Glutex Cu 90©) or a sixth (Labicuper©) of the amount of Cu in comparison with the Cu hydroxide [25].

Cu gluconate (containing 8% of Cu^{2+}) showed efficacy comparable to Cu hydroxide (containing 35% of Cu^{2+}) in vineyard trials for managing downy mildew [26]. Acylbenzolar-s methyl (Bion 50 WG©) also confirmed its efficacy in vineyard trials.

Several new tested Cu formulations or mixtures provided effective disease control, but their efficacy levels decreased when lower rates of Cu^{2+} were used, and this pattern was similar for different formulations. Nevertheless, some general conclusions should be mentioned:

- The level of downy mildew control decreases negatively and logarithmically with to Cu levels.
- There is a threshold of Cu necessary for effective control of downy mildew.
- Higher concentrations of Cu ($> 0.6\ g{\cdot}l^{-1}$) do not increase the efficiency of the treatment.

2.8 Technosoils or recovering soils. Measures to minimize the negative impacts of Cu in soils

The use of amendments is a promising strategy for recovering soils. The use of limestone is an effective strategy to reduce Cu availability and phytotoxicity that has been used for many years [27–29]. Limestone promotes the increase in soil pH, causing deprotonation of acidic functional groups of reactive soil particles. This increases cation exchange capacity (CEC) and Cu adsorption, decreasing bioavailability and potential uptake by plants. Grapevines grown in soil treated with limestone showed increased growth, dry matter yield and photosynthetic efficiency in young grapevines in parallel with a lowest Cu concentration in root tissues.

Also, compost and biochar could help in slightly moderate acidic soils, with some positive effects of Cu^{2+} reductions by liming. In general, organic soil

amendments could achieve similar effects of Cu^{2+} reduction than liming, but they might be more valuable because of their beneficial effects on physicochemical soil characteristics and decreased risk of soil erosion. Therefore, compost and biochar are promising solutions because usually are non-expensive treatments and, biochar go beyond a simple liming effect [30].

Nevertheless, depending on its characteristics, the addition of organic amendments can result in the opposite effect (mobilization of Cu due to its complexation with low molecular weight and soluble organic compounds) [27]. Thus, the use of this agronomic practice must be evaluated case by case to not deteriorate the already altered soil conditions. In its turn, biochar can overcome this problem due to its different mechanisms of Cu complexation. Also, the application of treated coal fly ash can be a solution, especially if mixing with compost, overcoming the potential problems of Cu leaching and availability that may arise from the application of the compost alone.

Pyoverdine (Pvd) is a bacterial siderophore produced by some *Pseudomonas* species that can bind Cu in addition to iron in the soil. Pvd is expected to alter the dynamics and the ecotoxicity of Cu in vineyard soils. Cu phytoavailability depends to a great extent on Cu complexation in soil pore water, the latter being highly sensitive to pH: vineyard topsoils with pH ranging from 5.9 to 8.6 can present Cu mobility differences of six times and, a Cu phytoavailability differing by a factor of 5000 among them. The Pvd action depends on Fe soil availability, the soil composition (e.g. carbonate soils more easily mobilized Cu) and other factors [28].

Besides, many several bacterial strains can hyper-accumulate and/or sequestrate Cu [27].

Another example is the mutualistic association between arbuscular mycorrhizal fungi (AMF) and plant roots that can minimize the toxic effects of Cu in plants, due to the complexation of this element with organic substance produced and released by them. Also, AMF can store Cu in cellular compartments such as vesicles and spores [27].

2.9 Modeling downy mildew

In the last decades, many epidemiological models have been elaborated to better manage fungicide application schedules. The correlations among environmental factors, host susceptibility and the pathogen have been well known for a long time: the so-called 3–10 rule (3 days under 10 mm or more effective precipitation) was the first attempt to predict primary infections of *P. viticola* [31]. Similar models have been developed in France [32, 33], Germany [34], USA [35, 36], and Australia [37, 38]. Unfortunately, they often fail to predict the real development of epidemics and their practical use is restricted [39]. Empirical models have shown some critical restrictions and limitations being too simple, due to the lack of robust cause-effect relationships in many model equations and therefore, requiring some corrections and calibrations to adapt to grape-growing areas or environmental conditions different from those used for the model development [40].

A mechanistic dynamic model was recently elaborated in Italy [41], which accounts for the biological effects of weather on the different stages of the primary infection chain, from the progressive breaking of dormancy in the overwintering oospore population to infection establishment during the grapevine-growing season. The model of Rossi et al. [42] was evaluated in more than 100 vineyards in Italy (from 1995 to 2007) as well as in the environmental conditions in the province of Quebec, Eastern Canada, by comparing the time of first lesion occurrence predicted by the model with field observations [42, 43]. This model always showed very high accuracy [44] and when used to schedule fungicide application against

downy mildew, allowed a reduction from 50 to 66% in fungicide applications, corresponding to an average saving of 174 and 224 €·ha^{-1}, respectively [42]. Finally, it was integrated into a DSS named vite.net® [45].

Moreover, Caffi et al. [46] developed a weather-driven model to predict *P. viticola* population dynamics on grape leaf surfaces during a discrete wet period. The authors positively correlated the post-inoculation efficacy of two cooper fungicides with the proportion of *P. viticola* sporangia on a leaf that had not yet caused the infection. Model simulations suggested that the efficacy of a copper treatment increased when the environmental conditions were less conducive for disease development. Therefore, this model can be used to predict whether a fungicide application during a discrete infection period will be effective [42].

2.10 Decision support system

To help growers optimize the scheduling and dosages of fungicides against downy mildew, decision support systems were developed based on weather data, disease risk, and plant growth [45, 47].

The DSS vite.net® is an Internet-based platform for sustainable vineyard management [41] that has two main components: (i) an integrated system for real-time monitoring of vineyard data, and (ii) a web-based tool that analyses data by using mechanistic and, dynamic models that can predict grapevine growth, risk of disease infection, and residual protection by the last fungicide application. Each of these models has been published and their accuracy validated [45–50].

The combination of site-specific weather data, monitoring reports and advice from a DSS enables growers to protect their vineyards by modulating the frequency and timing of copper applications, based on disease risk [51].

The DSS vite.net® was tested in 21 organic farms and allowed the reduction of copper applications by an average of 24%, and the total amount of copper applied by 37% compared to a calendar-scheduling of copper application that provided the same level of protection in organic vineyards, with an average saving of 195 €·ha^{-1}·year^{-1} compared to the common farm practice [52].

3. International legislation for PPPs application in vineyards

Regarding the international legislation, the aim of reducing pesticides in viticulture has been addressed by European and international bodies and organizations.

The International Organization of Vine and Wine (OIV) is an intergovernmental organization established under the Agreement of 3 of April 2001, which is directly related to a previous agreement (OIV Treaty, 1924) made for the creation in Paris of an International Wine Office.

OIV is an intergovernmental organization (47 countries), comprising scientific and technical knowledge in grapevines, wine and wine-based beverages, table grapes, dried grapes, and other vine-based products, with an international reputation and generally recognized competencies. OIV countries represent more than 80% of total world wine production, and, being present in main continents worldwide.

The principal objective of OIV is to contribute to the international harmonization of existing practices and standards and, if needed, to draft new international standards for grapevine and wine products. OIV is also cooperating strongly with international organizations intergovernmental or non-intergovernmental like *Codex Alimentarius* or World Health Organization (WHO) among others.

Under a proposal from one of its group of experts (Vine protection and viticulture techniques "PROTEC"), OIV wanted to suggest some recommendations or good practices for minimizing the impacts associated with the application of plant protection products (PPPs) in vineyards.

A questionnaire was launched between 2014 and 2015 to its Member States and, answers showed some relevant results. For example, all of them have an Official List for prohibited and allowed products for grapevine protection and almost all of them (90%), has an official methodology about applications limits [53].

This new resolution (VITI 592–2018[11]) includes some relevant points above described:

1. Methodology. Recommendations for the application of the PPP should be established based on the different factors that may help to determine the optimum volume of application (key factor, but not only) like Phenological stages of grapevines; Leaf area development; Varietal susceptibility to diseases suppressed; Climate and soil conditions; Training and trellising system; etc.

2. Products. Methods should define a specific limit for each product referring to the range among the treatments or doses used for it. It recommends undertaking (before its authorization) field trials and external audits given by official national departments or independent competent bodies. Pathogen resistance should be considered, and the product should be specific as possible for the intended target pest organism.

3. Doses. Quantities of PPP per hectare and treatment must be determined based on the volume or surface to be targeted or treated. Two models are strongly recommended: Tree Row Volume (TRV) or Leaf Wall Area (LWA) (Annex I).

4. Machinery for PPPs applications. General recommendations about the use of most efficient and environmentally friendly technologies for the vineyard treatments, like spraying or air-assisted sprayer techniques combined with injection nozzles or techniques which allow a homogenous application side by side and if possible, its recycling systems too (panels or other recovery systems). Calibrating procedures will be essential for the right dose rate adjustment. Drift Reduction Technology should be also encouraged.

5. Handling of plant protection products, training programs and national PPPs Plan should be drafted as guidelines for each member state.

The resolution was completed with five annexes with most used models, decision support systems (DSS), conversion factors and an official list from departments and websites related to PPPs national rules and recommendations.

Talking about the EU framework[12], some regulations should be considered, especially for organic production. As mentioned before, the rules for the implementation of organic production and labelling of organic products and control, describes quite well in article 5 and its Annex II. Pesticides — plant protection products, the use of Cu as fungicide up to 6 kg Cu per ha per year. For perennial crops, Member States may provide that the 6 kg Cu limit can be exceeded in a year provided that the average quantity actually used over 5 years consisting of that year and the four preceding years does not exceed 6 kg (it means 30 kg·ha^{-1} for 5 years

[11] https://www.oiv.int/public/medias/6450/oiv-viti-592-2018-en.pdf

[12] EC N° 834/2007

limitation). Cu can be applied under the form of Cu hydroxide, Cu oxychloride, (tribasic), Cu sulphate, cuprous oxide, Cu octanoate.

Recently, this limit was revised (based on some EFSA reports) and consequently, Cu compounds were designated as candidate substances for substitution and reduced applications, restricting the use of plant protection products containing Cu compounds to a maximum application rate of 28 kg/ha of Cu over 7 years (i.e. on average 4 $kg{\cdot}ha^{-1}{\cdot}year^{-1}$). This is described in clause 15 in the first statement (EC N° 1981/2018) and it has two annexes with the use and forms of Cu and their specific provisions. This regulation shall be applied until 2025 or previous revision.

It also is remarked that Cu sulphate was authorized in organic wine production until 31 July 2015 (EC N° 203/2012).

Therefore, within this framework, the research focused on real alternatives to reduce or substitute the Cu products, with other active principles or compounds for controlling the pest and diseases in grapevines are a key challenge for the sustainability of the wine sector.

4. Cutting edge lines from R&D ongoing projects developed by the wine sector

The Framework Programmes for Research and Technological Development, also called Framework Programmes (FPs), are funding programmes established by European Commission to support and promote research in the European Research Area (ERA). Since 1984, European Community research and technological development activities have been defined, implemented and founded by a series of multi-annual FPs (**Figure 1**)[13], getting close to €100 billion for the new Horizon Europe (2021–2027) and the Euratom Research and Training Programme.

Soil degradation is a global problem, often caused by several factors: unsustainable management and agricultural overexploitation practices, climate change, pollution, and deforestation. Soil degradation may intensify the impacts of natural disasters and contributes to social issues, (e.g. depopulation or migrations). The EU suffers from different levels of land degradation, and thirteen EU Member States have declared themselves as affected Parties under the United Nations Convention to Combat Desertification (UNCCD). The EU itself is one of the signatory members since 1998. Unfortunately, recently published studies and expert's opinions, released by the European Environment Agency's 2020 State of the Environment Report, the Special IPCC report on Climate Change and Land and the IPBES Assessment Report on Land Degradation and Restoration demonstrated that during the last years soils have been degraded dramatically at European and global level. In response, in May 2021 the EU announced a new Biodiversity Strategy for 2030. It adopts a comprehensive, ambitious, long-term plan for protecting nature and reversing the degradation of ecosystems, including a whole section dedicated to the soil.

It is expected that this new strategy will deliver a powerful tool to raise awareness on the importance of soils, engage citizens, create knowledge, and develop solutions for restoring soil's health and functions. Research and innovation are crucial to better understand, monitor and measure the specific effects of agricultural and forestry activities on soils and ecosystems functions. Transfer of knowledge and know-how are required to improve soil biological, chemical, and physical properties. Outstanding and breakthrough ideas are essential for achieving the objectives

[13] https://ec.europa.eu/info/sites/default/files/budget-may2018-research-innovation_en.pdf

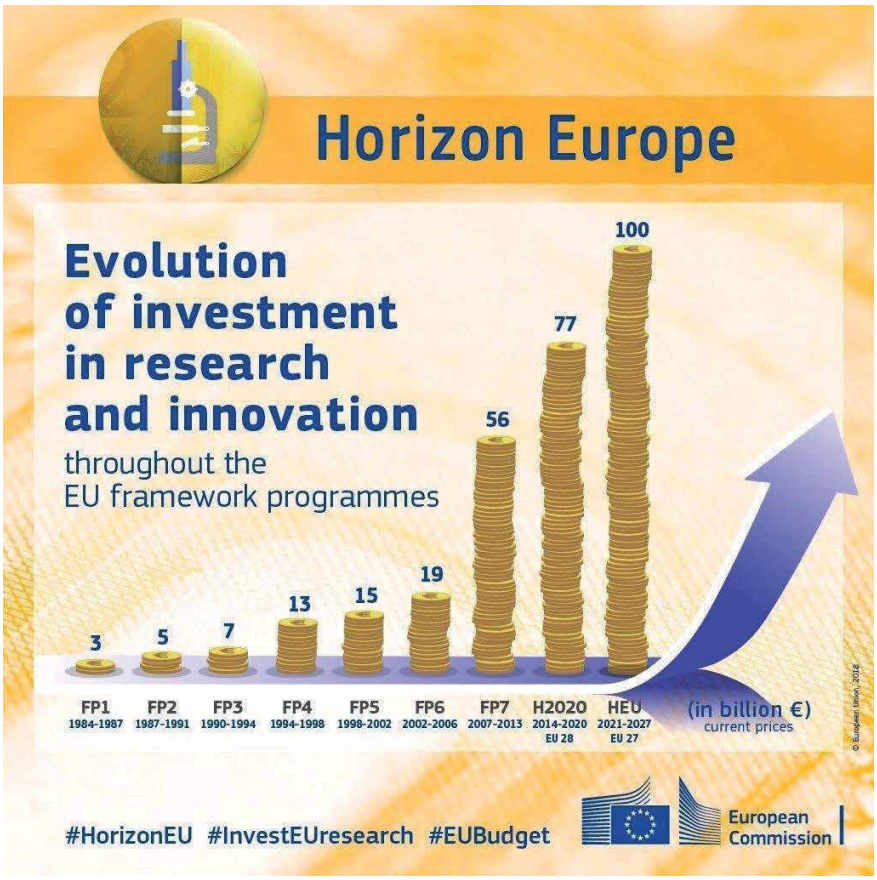

Figure 1.
EU framework programmes budget evolution.

of the European Green Deal, which is a set of policy initiatives of the European Commission with the overarching aim of making Europe climate neutral by 2050.

Horizon Europe is presently, the European Union's flagship Research and Innovation programme, part of the EU-long-term Multiannual Financial Framework with a budget of €95,5bn to spend over seven years (2021–2027). Previously, technological development and innovation in ERA have been carried out under the scope of project calls launched during the period 2014–2020 in the frame of Horizon 2020 (H2020). Indeed, one of the identified challenges of this H2020 program was named: "Food Security, Sustainable Agriculture and Forestry, Marine, Maritime and Inland Water Research and Bioeconomy". To achieve the objectives highlighted in this challenge, the European Commission, provided a budget of around 3.7 billion euros, out of which at least 1.5 billion euros were dedicated to carrying out research projects in agriculture and forestry.

Besides, during the H2020 8FP soils were the target of increasing political attention at European and global levels. The United Nations declared 2015 as the International Year of Soils, while the International Union of Soil Sciences at the Vienna Soil Declaration on Dec. 7th of 2015 proclaimed that 2015–2024 would be the International Decade of Soils.

In this context, and due to the serious environmental problems caused by the continuous use of Cu-derived phytosanitary products for decades, several projects to decrease/substitute Cu use in agriculture, have been granted within 7FP or 8FT (H2020).

Project acronym	Project title	Project duration	Project budget
COPPEREPLACE	Development and integral implementation of new technologies, products, and strategies to reduce the application of Cu in vineyards and remedy of contaminated soils in the SUDOE region	2020–2023	€ 1.638.340,72
NOVATERRA	Integrated novel strategies for reducing the use and impact of pesticides, towards sustainable Mediterranean vineyards and olive groves	2020–2024	€ 5.507.110,20
RELACS	REpLAcement of Contentious inputs in organic farming Systems	2018–2022	€ 3.999.675
BioAvenger	Biofungicide saves plants from fungal attacks.	2019–2019	€ 71.429
ProEcoWine	Development of a process to generate a novel plant protection product enriched with micronutrients to replace Cu in organic viticulture	2012–2014	€ 1.579.149,71
MicroWine	Microbial metagenomics and the modern wine industry	2015–2018	€ 3.945.597,12
DROPSA	Strategies to develop effective, innovative, and practical approaches to protect major European fruit crops from pests and pathogens	2014–2018	€ 8.602.632,24
WILDWINE	Multi-strain indigenous Yeast and Bacterial starters for 'Wild-ferment' Wine production	2012–2015	€ 1.592.302,40
CO-FREE	Innovative strategies for Cu-free low input and organic farming systems	2012–2016	€ 3.994.513,60
INNOVINE	Combining innovation in vineyard management and genetic diversity for a sustainable European viticulture	2013–2016	€ 8.489.665

Table 1.
Projects funded by European Commission aimed at promoting organic agriculture.

Table 1 highlights some of them, as well as their executions, which started in 2012. Besides the Framework Programmes, European Union (EU) has other instruments to fund projects with high impact on Regional development like Interreg programme, which supports cooperation across borders through project funding. The main aim of Interreg is to jointly tackle common challenges and find shared solutions in fields such as health, environment, research, education, transport, sustainable energy and more.

COPPEREPLACE [54] project, co-founded by the Interreg SUDOE programme, aims to develop and validate a series of integrated, innovative, and viable solutions to reduce the use of Cu and its environmental impact in vineyards. The solutions promoted within the project will be transferable and durable to allow the wine sector complies with the new European legislation and to promote environmentally sustainable production. **COPPEREPLACE** is led by the Wine Technology Platform (PTV) and has an international consortium comprised of Spanish, French and Portuguese entities: the Associaçao para o Desenvolvimento da Viticultura Duriense (ADVID), Institut Français de la Vigne et du Vin (IFV), Sogrape Vinhos, Centro de Valorización Ambiental del Norte (CVAN), Vignerons Bio

Nouvelle-Aquitaine (SVBNA), Eurecat, Família Torres, University of Porto and its Sustainable Agrifood Research Centre-GreenUPorto (Portugal), University of Vigo and Polytechnic University of Catalonia (Spain), LBS (Gérard Bertrand) and Jean Leon. In addition, the consortium has the support of Artica Ingeniería y Innovación (artica+i) consultancy. **COPPEREPLACE** will create a network of stakeholders that includes wine growers and other representatives of the international grape and wine-growing sector.

NOVATERRA project, funded by EC H2020 [55] with the main objective of reduction of the use and impact of pesticides used in Mediterranean vineyards and olive groves, while maintaining sustainable yields and quality of final products. Three are the pillars to achieve the goals: new natural plant protection products, smart farming techniques, (which include optimized spray applications, early detection of symptoms, decision support systems, and robotics) and soil management practices, enhancing functional biodiversity. These three pillars are being tested and analyzed in case of studies through Greece, Italy, France, Spain, and Portugal, under different conditions. Results will be analyzed by cost–benefit and impact analysis, final users' acceptance and adoption, consumers' willingness to pay, and validated by multidisciplinary stakeholders. Finally, new Integrated Pest Management strategies will be designed and disseminated aiming to reduce the environmental and health-related damages of food production.

The general objective of the H2020 **RELACS** project [56] is to foster development and facilitate the adoption of cost-efficient and environmentally safe tools and technologies, to phase out the dependency on and use of contentious inputs in organic farming systems. It is expected that the know-how generated under **RELACS** project will reduce the use of Cu and mineral oil, manure from conventional farms. As part of project deliverables, reports/technical descriptions defining alternatives to excessive use of anthelmintics in small ruminants, to reduce antibiotic use in dairy cattle, and moderate reliance on synthetic vitamins in cattle and poultry production were planned.

As it was mentioned in the previous sections, agricultural and horticultural industries need a way of dealing with fungal infections in non-chemical ways. The EU-funded **BioAvenger** [57] project begun the development of such an alternative. The project's prototype of the same name is a bio-fungicide for soil treatment. According to the project consortium, **BioAvenger** combated fungal infection in plants in a natural way. The product could be applied as either a cure or a preventative treatment. Obtained results demonstrated that in case the crop plants were sick, use of the treatment improved health within a month. Usual dosing over several months resulted in the eradication of over 90% of the invading fungi and up to 50% more plant growth. Unfortunately, the product is not yet developed or available in the market.

ProEcoWine project [58] set out to develop a novel, nutrient-enriched bio fungicide to combat common grapevine fungal diseases. Project partners successfully cultivated several microalgae species against downy mildew and Botrytis under different conditions. They screened the strains for antifungal activity and identified the two most capable microalgae strains with over 90% fungicide efficiency. The two strains and their antifungal activity were validated in a series of greenhouse and field experiments. The project team developed effective and economically viable methods for high microalgae density growth. They scaled up the production, processing, and storage of microalgae formulations for application as a fungicide. Researchers evaluated downstream methods required to activate microalgae antifungal activity to determine the most cost-effective process for product manufacturing. They established the ideal formulation of microalgae concentrate, resulting in products with enhanced

shelf life. It is forecasted that thanks to **ProEcoWine**, the innovative microalgae plant protection product will increase vineyard productivity by up to 30%, and decrease production costs per unit by up to 20%. This in turn will increase the competitiveness of EU wines and support the development of organic markets. The antifungal activity of the developed products was monitored and showed that the **ProEcoWine** products fully inhibited the presence of pathogens and had no adverse effect on plants (phytotoxicity).

The **MicroWine** [59] network was created to train a new generation of researchers with the aim to develop tools and gather knowledge for a modern DNA-based approach to European winemaking. It is expected that specialized scientists will transfer their knowledge to decrease the amount of Cu used in agriculture.

Investigations carried out under this project allowed uncovering microbial contributions to several phases of winemaking, from microbial influence on plant health to the microbial role in fermentation processes and influence on wine aroma and sensory perception and, seasonal microbial dynamics on grapevine leaves under biocontrol and Cu fungicide treatments [60].

The aims of the **DROPSA** [61] project was to developing reliable, robust, and cost-effective approaches to protect the major European fruit crops from *Drosophila suzukii*, and quarantine pathogens *Pseudomonas syringae pv*. *actinidiae* (Psa), *Xanthomonas fragariae* (Xf) and *Xanthomona arboricola pv. pruni* (Xap). They are identified as major phytosanitary risks and pose significant challenges to fruit production. The project consortium reported that pests and pathogens cause losses to the EU fruit industry of €10 billion and 3 million tons of produce. **DROPSA** addressed Cu problems advancing options beyond those currently available in the market according to secure food production lines in the EU.

From Greece and Spain to Germany and Romania, Europe already enjoys a strong winemaking tradition with a remarkable variety of flavors and bouquets. Nevertheless, modern winemakers generally use commercially available yeast and lactic acid bacteria (LAB) starter kits, leading to more homogenous European wines. One way to return to regionally distinct wines is by using locally occurring yeast and LAB species to create 'wild-ferment' terroir wines. With this in mind, the **WILDWINE** [62] project investigated regional microbial diversity to develop original starter cultures that can be used to make such unique wines. During the project, scientists analyzed several dozens or hundreds of *Saccharomyces* and *non-Saccharomyces* strains and a few tens of *Oenococcus* and non-*Oenococcus* bacteria. One of the project objectives was to investigate how the presence of Cu could influence fermentation processes.

The **CO-FREE** [63] project aimed to develop innovative methods, tools, and concepts for the replacement of Cu in European organic agriculture (grapevine, potato and tomato) production systems. The project promotes alternative compounds and, 'smart' application tools for integrating them into traditional and novel Cu-free crop production systems. Some strategies were identified to develop 'smart' breeding goals through crop ideotypes and by, fostering the acceptance of novel disease-resistant cultivars by consumers and retailers. The innovations and production systems were evaluated in a multi-criteria assessment concerning agronomic, ecological, and economic performance. In **CO-FREE** a total of 17 alternative compounds were studied for which modes of action, formulations, and application strategies were explored in the lab and field. As a major success of this project, one active substance was approved and included in the EC regulation 1107/2009, with other five dossiers submitted or being studied due to the efficacy of three additional alternative compounds, but additional R&D is still necessary. Most **CO-FREE** candidates exhibited safe ecotoxicological profiles in detailed studies on non-target organisms (beneficial arthropods, aquatic and soil indicator organisms).

Costs for registration, however, are high and require a substantial initial investment by small or medium enterprises (SMEs). This means that considering that (i) Cu has broad-spectrum activity, (ii) it is unlikely that only one compound isolated will have the potential to completely replace Cu in all crops, (iii) the alternative compounds, at the best, will have similar efficacy as Cu, and (iv) the new compounds have to remain effective over time, several different candidate compounds are likely necessary to further reduce/replace Cu. **CO-FREE** has thus, contributed strongly with several candidate compounds with a technology readiness level of 8, which provided the foundation for the development of new products for the market.

INNOVINE [64] project globally led to a better understanding of the impact of vineyard practices and various abiotic stresses on grapevine physiology and berry composition in the context of climate change. The development of two grapevine models allowed us to simulate and predict those impacts in various climatic scenarios. Further models' implementation had to be addressed, taking into account differential impacts on different genotypes. Methods for screening germplasm for plasticity or for identifying key molecular pathways of adaptation to stress were proposed. Several non-destructive phenotyping tools based on fluorescence, reflectance, thermal imaging and or, hyperspectral imaging were experimented and validated in several work packages of **INNOVINE** to monitor the physiological status of the canopy, as well as the berry content or the onset of downy mildew attacks. Researchers from different scientific areas developed a foreground that allowed them to carry out strategies for sustainable control of diseases in the vineyards. The most important level for the diminution of pesticides was found to be the use of resistant varieties. A very important effort was carried out for the screening of yet uncharacterized germplasm collections for resistance to diseases and was made available through publication in papers and the European Vitis Database. However, it was also shown that the populations of downy and powdery mildews could slowly adapt to resistant varieties and overcome these resistances. The current disease models were improved to consider grapevine physiology and genetic diversity. Finally, **INNOVINE** showed that canopy management practices impact the berry size and therefore, the *Botrytis* incidence.

5. Conclusions

Even if several (R&D) projects have been developed in recent years, replacing or giving alternatives to the use of Cu in viticulture, this problem is still currently unsolved, being one of the most relevant challenge for the wine sustainable production. Before providing or modifying some new standards or rules, results from these projects should be considered to not compromise sustainability targets in viticulture, assess the present risks of using Cu in viticulture and to better support establishing limits for its applications.

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Author details

Mario De La Fuente[1*], David Fernández-Calviño[2], Bartosz Tylkowski[3], Josep M. Montornes[3], Magdalena Olkiewicz[3], Ruth Pereira[4,5], Anabela Cachada[4], Tito Caffi[6], Giorgia Fedele[6] and Felicidad De Herralde[7]

1 UPM-Polytechnic University of Madrid, Madrid, Spain

2 Faculty of Science, Department of Plant Biology and Soil Science, UVIGO-University of Vigo, Ourense, Spain

3 Eurecat, Centre Tecnològic de Catalunya, Tarragona, Catalunya, Spain

4 Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Porto, Portugal

5 GreenUPorto, Sustainable Agrifood Production Research Centre, Edifício de Ciências Agrárias (FCV2), Vairão, Portugal

6 Department of Sustainable Crop Production (DI.PRO.VE.S.), Università Cattolica del Sacro Cuore, Piacenza, Italy

7 IRTA-Institute of Agrifood Research and Technology, Caldes de Montbui, Spain

*Address all correspondence to: mario.delafuente@upm.es

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Chapter 4

Artificial Intelligence and Big Data Analytics in Vineyards: A Review

Nathaniel K. Newlands

Abstract

Advances in remote-sensing, sensor and robotic technology, machine learning, and artificial intelligence (AI) – smart algorithms that learn from patterns in complex data or big data - are rapidly transforming agriculture. This presents huge opportunities for sustainable viticulture, but also many challenges. This chapter provides a state-of-the-art review of the benefits and challenges of AI and big data, highlighting work in this domain being conducted around the world. A way forward, that incorporates the expert knowledge of wine-growers (i.e. human-in-the-loop) to augment the decision-making guidance of big data and automated algorithms, is outlined. Future work needs to explore the coupling of expert systems to AI models and algorithms to increase both the usefulness of AI, its benefits, and its ease of implementation across the vitiviniculture value-chain.

Keywords: Artificial Intelligence, Big data, Climate change, Decision support, Expert knowledge, Vitiviniculture, Risks

1. Introduction

Viticulture is at the front line of climate change as grape production is highly sensitive to changing environmental conditions. Growers, producers, and investors plan and anticipate risks far into the future with long time horizons (i.e., 7–11 years or more) for investing, establishing, and attaining positive net income and returns on investment. Growers are grappling with unpredictable, rapidly changing weather patterns and more frequent and intense extreme events such as spring frosts, floods, droughts, heatwaves, and wildfires. Seasonal climate changes of hotter and longer summers and warmer winters are shifting areas suitable for growing grapes further north in the Northern Hemisphere (NH), and south in the Southern Hemisphere (SH), from historical cultivation latitudes of 4° and 51° (NH) and 6° and 45° (SH) [1]. This is driving wine makers to move vineyards to higher elevations that provide colder nighttime temperatures and less frequent and intense peak daytime temperatures to ripen grapes, while preventing over-ripening [2, 3]. Climate change warming scenarios project that grape cultivar diversity may buffer wine-growing regions from losses resulting from both the reduction of suitable areas for growing grapes and attainable yields. In a recent global study using data on long-term French records to extrapolate globally for 11 cultivars (varieties), increasing cultivar diversity more than halved future, projected losses of current wine-growing areas and decreasing areas lost (56 to 24%) under a 2°C warming scenario, and reducing areas lost by a third (85% versus 58%) under a 4°C warming

scenario [4]. These warming scenarios combine daily temperature and precipitation from a large ensemble of the Community Earth System Model (CESM), alongside winegrape phenology and global variety-level planting data [5, 6], projecting geographical shifts of areas suitable for grape varieties as well as phenological shifts in the timing of grape ripening (veraison). The resulting loss of suitability of areas is primarily attributed to shifting temperature regimes, and greater accumulations of temperatures above 25°C, and number of days above 40°C. Precipitation was found to have a buffering effect, both reducing the number of varieties that were lost over time, while increasing the capacity for cultivar turnover [4]. While growing diverse cultivars that are more heat-tolerant and drought-resistant can reduce area and yield loss due to climate change impacts, the industry still faces the uncertainty and complexity associated with fulfilling the stringent consumer demands for quality, novelty, cost and sustainability of this agricultural product.

Big data (BD) is data that is machine-readable as opposed to human-readable. There is no official size that makes data "big". It consists of massive amounts of digital information, collected from all sorts of sources that are too large, raw, or unstructured for analysis using conventional relational database and techniques. The internet-of-things (IoT) (i.e., the network of physical objects that exchanging data between devices, software, and systems over the Internet) continues to create BD and expand globally. Artificial intelligence (AI) refers to the simulation of human intelligence in machines that are programmed to think, learn and problem-solve like humans and mimic their actions. Machine learning (ML) is a sub-set of AI where machines learn from data without being explicitly programmed. Deep learning (DL) is a subset of ML in which artificial neural networks (ANNs) mimics the structure of the human brain, to adapt and learn from vast amounts of data. Algorithms are procedures that are implemented in computer code that use data, and are, in general, distinguished from models, which comprise many algorithms. BD needs to be of sufficient high quality to reliably train, validate, and independently test and/or reproduce algorithmic and model output at reported levels of accuracy and reliability. Here the goal is to design AI algorithms with a fast and efficient learning speed, fast convergence to a solution, good generalization ability and ease of implementation.

2. Review objective and methodology

This review explores the benefits and challenges of BD and AI to sustainable viniviticulture through the lens of recent research findings and insights. Detailing all the different AI methodologies and their implementation is beyond the scope of this review that focuses on their domain application. For background reading of state-of-the-art AI methods and solution techniques, we direct interested readers to an article that features how vineyards are making use of BD [7], a recent introductory methodological reviews of ML in agriculture [8], and DL [9]. In the review conducted and reported here, recently published and highly relevant scientific journal articles were searched and selected using the University of Victoria (UVic)'s Summons 2.0 search engine, which includes a wide range of scientific databases, including the Scopus, ScienceDirect and PubMed databases. A total of 59 articles were selected that met the required, minimal criteria that they assessed, applied, adapted, or developed an AI method/algorithm and addressed a main aspect linked with viniviticulture. This search approach was selective rather than exhaustive or systematic. The resulting sample size is similar to the 40 articles selected as part of another recent AI review which also employed online search of major scientific databases [8].

A systems overview of vitiviniculture interactions and drivers of change was first constructed. This was used to distinguish 10 major aspects under which a range of use-cases could be identified and linked across the selected works. This was informed, in part, by a broad review of vineyard ecosystems, their multifunctionality, and ecosystem services, applied the Common International Classification of Ecosystem Services (CICES) highlights the need to better identify and understand interactions within vineyards, identifying six ecosystem services (or aspects) that are most studied, namely: i) cultivated crops, ii) filtration and sequestration, iii) storage and accumulation, iv) pest and disease control, v) heritage and cultural services, and vi) scientific services (e.g., studying vineyard agronomy) [10]. Challenges identified and described within the selected articles were next extracted, compiled, and synthesized into a summary Table. A depiction or simplified design of a novel BD value chain informed by an ES comprising expert knowledge and providing an ES system with an ability to learn is presented. This is structured to encompass all the identified aspects and potentially capable of addressing current research challenges.

3. AI in Vitiviniculture

Viticulture is at the front line of technological disruption driven by automated, AI algorithms that integrate and learn from large complex data obtained from diverse sources both old and new. New technologies and data sources include satellite and drone remote-sensing, field sensors, and automated weather stations which are increasingly being deployed and used to enhance decision-making because of their increased availability, affordability, and reliability. For example, Palmaz vineyards in California's Napa Valley are early-adopters of BD and AI, bringing innovation and invention to the ancient art of making wine. They use monitoring and geospatial technology for guidance and decision support. This includes VIGOR (Vineyard Infrared Growth Optical Recognition) to monitor and adjust conditions in the vineyard and an intelligent wine-making assistant, FILCS (Fermentation Intelligent Logic Control System), nicknamed Felix, and STAVES (Sensory Transambiental Variance Experiment) to monitor wines as they age in the barrel [11]. New decision-support tools have also been developed that use BD and AI technology provided by Sippd^{TM} and Vitiapp^{TM} [12, 13]. There are aspirations even to build an AI system (i.e., a Turing AI taster) that can out-perform a wine expert? [14]. Sippd offers a commercially-available, personal sommelier that uses AI to help consumers discover wines based on taste and budget, with personalized wine recommendations. VitiApp^{TM} is a pre-commercial web-based application for supporting decisions about vineyard management. It includes environmental data (weather, soil) to describe conditions influencing grape yield and fruit composition, cloud computing to integrate multiple data streams from a diversity of vineyard sensors and weather forecast data. It provides vineyard patch-specific awareness of weather-based risks for each selected management issue: botrytis/powdery/downy disease, and frost/chilling/heat accumulation, wind, rainfall, soil moisture and/or spraying conditions.

While often used interchangeably, viti-culture refers to the science, study, and production of grapes, whereas vini-culture is specific to grapes for winemaking; when combined is vitiviniculture. According to the International Organization of Vine and Wine (OIV), sustainable vitiviniculture is a â€œglobal strategy on the scale of the grape production and processing systems, incorporating at the same time the economic sustainability of structures and territories, producing quality

products, considering requirements of precision in sustainable viticulture, risks to the environment, products safety and consumer health and valuing of heritage, historical, cultural, ecological, and landscape aspectsâ€ (see [15] and references therein). While sustainable wines are currently a niche market, they are increasing in number, and consumers are willing to pay a premium for sustainably produced wines. Actions and guidance need to incorporate uncertainty and be fine-tuned to the local conditions and impacts. Grapevines phenotype (terroir), canopy microclimate, vine growth and physiology, yield, and berry composition all contribute various attributes to wine and the degree to which it reflects its varietal origins and signature characteristics or typicity [1]. Vitiviniculture management is likely to become more complex. There are also stringent rules and regulations linked with production certification schemes and labelling systems for vineyards that apply organic, sustainable, biodynamic practices that include reducing environmental risks. The Summerhill Pyramid Winery based in Kelowna, British Columbia, Canada, for example, was certified in both organic under Canadian organic standards (PACS # 16-077, COR Section 345) in 1988 and Demeter biodynamic certification in 2012. Timely, suitable, and cost-effective adaptation strategies and enhanced foresight are crucial to support the complex dynamics and management of vitiviniculture.

4. AI learning algorithms and model types

There are three main types of learning: *supervised* that learns known patterns, *unsupervised* that learns unknown or hidden patterns, and *reinforced* that learns rules or actions in data to learn a pattern or decision process and can be value-, policy-, or model-based in how it optimizes its solution to a given complex problem. Classification and regression problems are supervised, clustering and anomaly detection are unsupervised. Learning algorithms differ according to the problem and their ability to be trained on different types and amounts of data without being overfitted. Overfitting is a concept in AI and data science, which occurs when a statistical model fits exactly against its training data because it memorizes the noise and fits too closely. Deep double descent is the phenomenon where performance improves, then gets worse as the model begins to overfit, and then finally improves more with increasing model size, data size, or training time. Essentially, there is a given level of complexity where models are more prone to overfitting, but if enough complexity is captured in the model, the larger the model and data, the better. Learning can be sequential, in which one part of a task is learnt before the next, or incremental, in which an algorithm learn from scratch and gradually obtains more knowledge with an increasing amount of training inputs or examples by adjusts weights of an observation based on the last classification. How algorithms are trained on data differs as well. Bagging (i.e., bootstrap aggregating) generates additional data for training a model by resampling a given dataset through repeatedly re-combinations to produce multi-sets of the original data. Learning can also be ensemble-based (termed batch learning or stacking) that combines several base models in order to produce one optimal predictive model. Bagging is suitable for high variance, low bias problems, boosting is suitable for low variance, high bias problems, and stacking combines different models to learn some parts of a problem, in solving the whole space of a complex problem. Popular ML algorithms differ in terms of how they find solutions and partition a given problem space. A Support Vector Machine (SVM) uses hyperplane partitioning, Random Forest (RF) uses tree-based ensemble partitioning, and Gradient Boosting (GB) use an ensemble of weak prediction decision trees. Adaboost or Adaptive Boosting assigns higher

weights to incorrectly classified data and Stochastic Gradient Boosting uses statistical bootstrapping of data to generate samples for implementing boosting. XGBoost is a boosting algorithm that benefit from 'regularization' that penalizes various parts of the algorithm to improve its performance by reducing overfitting.

ANNs comprise a collection of connected units or nodes called artificial neurons aggregated into different layers which transmit and process signals between their connections (edges). The signal of a given node is prescribed by a mathematical 'activation' function. Signals travel from a first 'input' layer, through one or more intermediate or 'hidden' layers, to an 'output' layer. Nodes in the hidden layer have values that are unknown and determined mathematically from their input and output signals as a network learns. Different layers may perform different transformations on their inputs. Connections can exist between nodes in different layers or between nodes within a given layer. Feedforward neural networks (FNNs) are a type of ANN having no memory, whereby signals only move in one direction from the input through to the output layer, never being processed by a node more than once. An extreme learning machine (ELM) is a FNN with a one or many hidden layers whose nodes can signal randomly, never update, or inherit previous signals without requiring any tuning of the mathematical function parameters of its node activation functions, or the weight values that alter the strength of how its inputs are connected within the network. A wide range of different DL model structures have evolved from FNNs. Recurrent neural networks (RNNs) are FNNs with memory whose nodes process signals in loops/feedbacks/cycles that considers current inputs and also what it has learned from previous inputs. Long-short-term-memory (LSTM) are a type of RNN that uses special units that include a â€¯memory cellâ€™ that maintains information in memory for longer periods of time. Convolutional neural networks (CNNs) have several layers whose nodes are sparsely connected (i.e., nodes are not fully connected) whose flexibility is particularly useful for image recognition and object classification. A CNN typically comprises four types of layers, namely, the convolution layer, rectifier (ReLU) layer, pooling, and fully connected layers. Every layer has its own functionality and performs feature extractions and discovers hidden patterns in input data. RNNs can use sequential information, while CNNs cannot.

Restricted boltzman machines (RBM) consist of a two-layer network of fully connected nodes with both forward and backwards connections (i.e., a cycle) that can share weights (i.e., bidirectional). This two-layer network was originally designed to better determine good starting weights (i.e., pretraining) of FNNs. A deep belief network (DBN) consists of RBMs which are sequentially connected, comprising multiple hidden layers, with connections between hidden units are in separate layers. Deep q-learning networks (DQLNs) use reinforcement learning to make a sequence of decisions through trial and error within an interactive environment involving 'agents' that have 'states' that change, learn, and adapt over time. Q-learning is a specified form of reinforcement learning (i.e., values-based learning) that is model-free i.e., does not require a model of the environment. It learns expected values of future rewards for actions of agents that are in a given state with a given 'value'. It uses q-learning (i.e., learning from delayed rewards) based on Bellman's Equation that decomposes the value of an agent's state into an immediate reward and the value of a cumulative set of successor states according to a discount factor that determines the importance of future rewards. Bayesian learning (or belief) networks (BLNs) are a type of network model that is stochastic or probabilistic and involves 'priors'. Prior is short for 'prior probability distribution' and is the probability distribution that express one's beliefs about an uncertain quantity before some data or further evidence is taken into account. They are used to represent spatial or temporal dependence (represented by conditional probability distribution

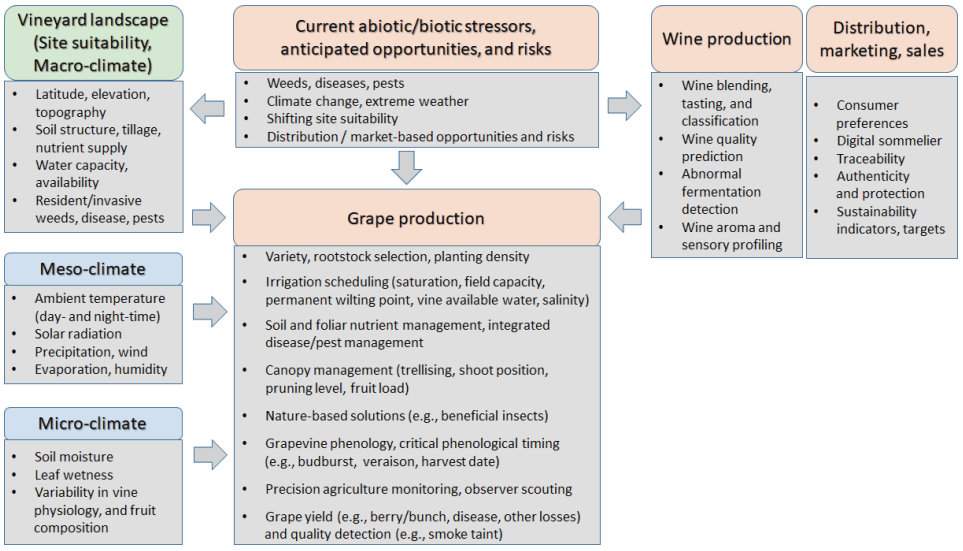

Figure 1.
Overview of the interactions of major climate, biotic, and abiotic drivers, stressors, and risks within vineyards.

functions) between multiple stochastic variables (i.e., nodes), describing how the variables depend on each other in terms of cause-and-effect or causality (i.e., connections or arcs between nodes). Variables can be discrete or continuous. BLNs can be prepared by experts or learned from data, then used for inference to estimate the probabilities for causal or subsequent events. Copula bayesian networks (CBNs) use a tailored mathematical function called a copula that provides an efficient way to represent and compute the joint probability represented by such networks along with how its variables depend on each other.

New methods and frameworks to use and integrate BD and AI for complex problem-solving and enhanced decision making will, very likely, be needed to support sustainable vitiviniculture. Such approaches will need to consider complex interactions between climate, biotic, and abiotic drivers, stressors, and risks within vineyards, influencing grape and wine production, and value-chain resiliency and sustainability (**Figure 1**).

5. AI use-cases and knowledge gaps

Structured data is highly organized and easily understood by machine language, whereas unstructured data is often categorized as qualitative data that cannot be processed and analyzed using conventional tools and methods and includes text, video files, audio files, mobile activity, social media posts, and satellite imagery. BD can include also vague and imprecise information, qualitative data, and rule-based logic. An expert system (ES) is a computer program, model, or algorithm that uses AI to simulate the judgment and behavior of a human or an organization that has expert knowledge and experience in a particular domain or field. It provides supervision for AI algorithms by human experts termed human-in-the-loop (HITL), whereby a model requires human interaction and intervention and is not fully automated or self-reliant. AI in winemaking based on an ES approach was explored in 2000 [16], with limited research on ES, and closely associated, fuzzy inference systems (FIS) in viniviticulture. Fuzzy theory and FIS represent vagueness and imprecise information often used in making decision in a mathematical way using

fuzzy sets and rule-based logic. Several leading examples are noteworthy. An ES for automated forecasting of optimal grape ripeness dates using data gathered from a vineyard wireless sensor network (WSN) has been developed and tested, but uses the Holt method (exponential adaptive forecasting for trended data) instead of ML or DL models/algorithms [17]. Also, an FIS that enables automating the classification of grape quality at harvest for grape growers has been developed and tested [18]. An ES for evaluating the sustainability of vineyards based on their management called Vigneto uses a fuzzy logic indicator [19]. A decision support system called FGRAPEDBN that uses fuzzy logic and expert knowledge is able to predict grape berry maturity. Berry maturity is measured as sugar concentration that increases rapidly, and acidity concentration, that decreases along with pH levels as berry mature. This ES attains high predictive accuracy (i.e., a root-mean-squared-error (RMSE) of 7 g/l (i.e., 0.44 g/l or 0.11 g/kg) [20]. The coupling of ES to AI (i.e., ML and DL models/algorithms) in viticulture, or agriculture in general, is still unexplored and in its infancy. Also, ES systems generally have no ability to learn decision rules, so could benefit also from being informed by AI/ML analytics and predictive insights.

A wide array of applications and use-cases of AI in vitiviniculture are evident, and are summarized in **Table 1**. This shows that there is substantial interest, applied expertise, and future potential in developing such approaches to help mitigate and adapt to climate change, address inter-related risks, and enhance decision-making and foresight. Current AI work is, however, concentrated heavily on grapevine yield prediction and grape variety classification using on the pattern recognition, detection, counting, and clustering of grape berries and bunches in imagery collected by observers, unmanned aerial vehicles (UAVs), and/or robots. Such imagery differs based on vineyard environmental conditions and grape variety altering illumination, occlusions, colors and contrast in images. Existing research limitations and challenges point to the need for robotics and mobile sensing platforms, the combination or fusion of both fine-scale hyperspectral and coarser-scale multispectral imagery data, as well as spatially-distributed sampling within vineyards to better measure and assess micro-climate variability linked with meso- and macro-climate and landscape suitability requirements that are changing with climate change.

Suitability requirements for vineyards would benefit from other AI/ML techniques to explore geospatial data and cross-validate geographical locations determined from CNN models applied to identify vineyards in satellite data. A wide range of different models for disease and pest control (i.e., a hybrid BLN, CNN, RF, GB) have been applied, and these multiple AI approaches could be coupled to provide a fully-integrated solution for processing field imagery, conducting data mining and analytics, and forecasting of disease risk in vineyards. Vineyard management is already exploring decision rule applications via case-based reasoning, and sequential methods of AI, but in isolation, and such work could greatly benefit from being coupled together to accelerate advancement. This would enable them to be tested on a broader set of vineyard data and to better identify best management practices, rather than a more incremental, siloed approach. Much more work is needed to explore opportunities and potential of BD and AI in vineyard biotic and abiotic factors and stress. Only a handful of studies have explored the use of satellite remote-sensing (i.e., Earth Observation or EO) data for detecting and mapping water and heat stress, yet large amounts of data for training and validating AI models now exists from EO data centers and providers. This could help to validate whether satellite indices can reliably detect and map stress variability in vineyard, what data fusion and satellite indices perform best, to port such BD and capabilities to support stakeholders proactive decision making ahead of extreme weather

Aspect	Use-cases	Method/ algorithm	Current challenges	References
Suitability requirements	detect, segment vineyards	CNN	spectral distortions dependent on wavelength, image acquisition parameters	[21, 22]
Grape/grape bunch detection	non-invasive, automated cluster compactness, variety discrimination, classification, tracking	DNN, CNN, AdaBoost and RWNN, SVM, ANN	high-quality training and validation data (different varieties, illumination conditions)	[23–31]
Disease and pest control	disease forecasting, automated detection and differentiation of diseases from leaf images	hybrid BLN, CNN, RF, GB	vineyard data on grape yield, disease imagery to validate models for different varieties, diseases, vineyards, climatic zones; deploying imaging systems on ground vehicles	[32–35]
Vineyard management, grape growing	automated grape vine pruning; irrigation, nutrients	RNN with LSTM, Case-based reasoning (CBR)11	learning rules of expert pruners; broader method testing; including inter-annual variability due to weather, climate;	[36, 37]
Biotic factors and stress	automated insect trapping; rhizogenesis and acclimatization; soil microbial biomass	ANN, genetic algorithm	expanding training data and introducing more parameters regarding soil physical properties and management	[38–40]
Abiotic factors and stress	water stress from hyperspectral imagery; heat stress from Sentinel-2 multispectral imagery	RF, EGB	classification using the widely-applied Savitzky–Golay smoothed spectra reduces accuracy	[41–43]
Grapevine phenology detection, yield prediction	grape berry maturity, yield prediction	fuzzy logic, dynamic BLN	reducing uncertainty with an integration of expert knowledge	[20, 44–48]
Wine aroma, sensory profiling	vertical vintage using near-infrared spectroscopy (NIR); weather/management data	Clustering, GO	coupling models to data using new and emerging technologies to make these analyses more affordable and user-friendly	[49, 50]
Wine quality, classification	wine preferences from physicochemical properties, organic acids; abnormal fermentation detection; wine blending, AI consultant; preference prediction	ANN,SVM	greater use, adoption of novel models/tools, cost–benefit analysis	[12, 14, 51–55]
Traceability, authenticity, protection	incident handling in wine storage; authenticity assessment; wine aging prediction; constructing wine barrels, smoke exposure	clustering, dimensional reduction	greater use, adoption of novel models/tools, cost–benefit analysis	[56–62]

Refer to abbreviation list for model/algorithms.

Table 1.
Showcase of AI/ML in vitiviniculture (partial set from the review).

impacts like heatwaves. Most work on wine aroma and sensory profiling still employs traditional statistical techniques and clustering with limited work on global optimization (GO). While decision tools already exist in the market to track the wine preferences of consumers, they could be better informed from AI analysis and prediction that links more objective, scientific data on new varieties, wine constituents, alternative wine blends and new wine grown in newly establish vineyards in more suitable areas as climate change shifts grape and wine suitability. The application of BD and AI in traceability, authenticity, and protection also relies on more traditional statistical methods, rather than BD and AI. This is surprising and was not expected before conducting this review, as this area involves large extents of the value-chain and major business risk. Here, government could play a vital role to co-design and pilot test new solutions alongside experts in BD and AI, as developing broad-based solutions in this aspect likely require broad collaboration, multidisciplinary expertise, substantial BD collection and sharing, and industry wide involvement, adoption, and deployment.

6. Proposed BD and AI framework

An existing ontology framework called the Agri-Food Experiment Ontology (AFEO) has been developed to guide the integration of data in a way that provides researchers with the information necessary to address extended research questions [63]. It contains 136 concepts spanning viticulture practices, wine-making products, and operations. It utilizes the Resource Description Framework (RDF) format, a standard model for relational data queries, interchange, and metadata processing, to represent these data in a standard format. Based on this review, an analytical framework is proposed that integrates BD analytics and AI prediction as part of a BD value-chain using expert knowledge as HITL intervention and guidance is outlined in **Figure 2**.

BD is distributed across different remote-sensing platforms (e.g., drone and satellite), across vineyards (e.g., networks of AI and climate-smart vineyards), and within vineyards (e.g., field sensor networks), and across data centers and

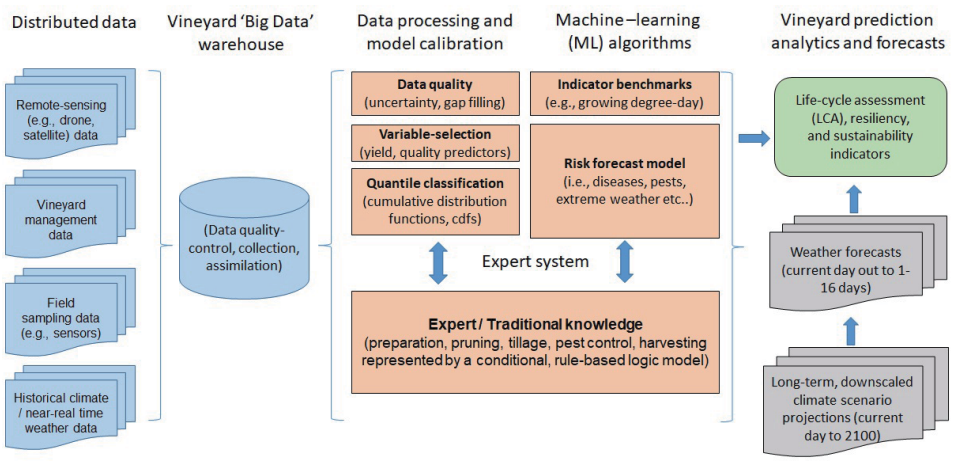

Figure 2.
Depiction of a vineyard BD value-chain that incorporates diverse, distributed vineyard data alongside an expert system. This system integrates traditional, cultural perspectives, knowledge, and reasoning of grape growers, viticulture specialists, and other wine industry stakeholders.

providers (e.g., long-term climate stations and weather monitoring networks providing both historical climate and near-real-time weather station data). Using a distributed cloud approach, an application of cloud computing technology, BD can be interconnected with public and private applications served from varied geographical locations for preprocessing quality control, data quality checks, model identification (i.e., variable selection, quantile classification), indicator model benchmarking, and the development of risk forecast models using AI. An ES system comprising conditional, decision rules provides traditional and expert knowledge, while informing AI model training and validation. An AI model then also learns by selecting rules from the master ES ruleset, adjusting and updating rules as it learns. In this way, the framework is agile and scaleable to address a wide range of stakeholder needs along the value-chain. This includes life-cycle assessment (LCA), providing data to support monitoring and tracking of vineyard sustainability indicators, and providing forecasts (i.e., foresight) to better anticipate future impacts, having additional lead time to mitigate and safeguard operations in time, and deciding between different possible actions and interventions to climate change (i.e., irrigation needs and limitations, disease outbreaks, extreme weather events) risks for more informed vineyard management scheduling and planning. Weather and climate transformed into tailored information and knowledge that vineyard stakeholders and users need and require are provided through customized Climate Information Services (CIS) help to drive forecasts of relevant vineyard indicators. This could integrate sub-seasonal and seasonal forecasting, alongside longer-term, downscaled inter-annual and decadal scenario projections. The quantification of risk (i.e., levels and associated uncertainties) is essential to determine an appropriate response. With an approach that can be scaled up to the entire vitiviniculture value-chain the adoption of BD and AI can be accelerated. This would enable all stakeholders to co-learn and collaborate in evidence-based and model-tested design tactics and strategies. Such an approach can ensure mitigation and adaptation actions and interventions are enabling, rather than inhibiting, to maximize perceived benefits and organizational readiness, while minimizing external pressures [64].

7. Conclusions

Vineyards that are certified organic and biodynamic, however, are not necessarily the same ones that are early- or significant-adopters of latest BD and AI technology that can accelerate and support the wider transformation from conventional to sustainable vitiviniculture practices. As discussed, this is because of a disconnect that exists between the path to adoption of sustainable practices and the path to adoption of BD and AI technology. This could be addressed by providing a way to structure and integrate an expert knowledge and insights from all stakeholders into an ES embedded within an overarching analytical framework. The majority of research challenges identified in this review, which span a wide range of aspects of viniviticulture, also point to the need for including expert knowledge to provide context and rules to design AI algorithms and their automated learning, while helping to structure data, obtain high-quality data for training AI models, and validate the use and adoption of new BD types and sources. Aligning the existing AFEO ontology that links vitiviniculture objects and experimental activities to an analytical BD and AI modeling, could accelerate the advancement of sustainable vitiviniculture. This would also provide the ES methodology with an ability to learn from experience which most systems cannot do currently. ML and DL models and algorithms need to be trained and informed by an ES that integrates imprecise and

vague information as well as qualitative data and decision rule-based logic that is used in stakeholder decision making. This will require linking the scientific and expert knowledge on climate and weather risks pertaining to drivers and interactions, the BD value chain, to address the identified research challenges outlined here. Future work will aim to synthesize knowledge and insights from the wide array of applications of ES, to design a representative ES for the proposed BD value chain.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

AI	Artificial intelligence
ANN	Artificial neural network
AFEO	Agri-Food Experiment Ontology
BLN	Bayesian learning network
BD	Big data
CBR	Case-based reasoning via a learning-based adaptation strategy
CESM	Community Earth System Model
CICES	Common International Classification of Ecosystem Services
CIS	Climate Information Services
CNN	Convolutional neural network
CBN	Copula bayesian network
DL	Deep learning
DQLN	Deep q-learning neural network
DSS	Decision-support system
EGB	Extreme gradient boosting via second-order derivative approximation (XGBoost)
EBM	Extreme learning machine
EO	Earth observation
ES	Expert system/s
FIS	Fuzzy inference system/s
FNN	Feed-forward neural network
GB	Gradient boosting via gradient decent
GO	Global optimization (constrained)
HITL	Human-in-the-loop
LCA	Life-cycle assessment
LSTM	Long short-term memory architecture
ML	Machine learning
IOV	International Organization of Vine and Wine

IOT	Internet-of-things
RDF	Resource Description Framework
RF	Random forest ensemble learning
RMSE	Root-mean-squared-error
RNN	Recurrent neural network
RBM	Restricted boltzman machine
RWNN	AdaBoost and random weight neural network
SVM	Support vector machine
UAV	Unmanned aerial vehicles
WSN	Wireless sensor network

Author details

Nathaniel K. Newlands[1,2]

1 Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, British Columbia, Canada

2 Department of Geography, University of Victoria, Victoria, British Columbia, Canada

*Address all correspondence to: nathaniel.newlands@canada.ca

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Chapter 5

Biological Characteristics of Native Grape Cultivars of Crimean Region and Availability of Their Use in Breeding

Svetlana Levchenko, Irina Vasylyk, Vladimir Volynkin, Vladimir Likhovskoy and Alla Polulyakh

Abstract

In the context of the global climate change, manifested in a rapid increase in environment temperature and a constant increase in freshwater deficiency, the problem of breeding new grapevine cultivars that would correspond to the present-day biosphere conditions emerged. The endurance of native cultivars to adverse soil and climatic conditions and their drought tolerance are of particular value in development of generative breeding. It is known that most of the Crimean native cultivars have a functionally female type of flower, low resistance to biotic environmental factors that affects the stability of fertilization, yield and directly depends on the climatic conditions of cultivation. The adaptive ability of Crimean native grape cultivars is possible to increase by method of hybridization. So, the specific objectives of the study include, definition of agrobiological parameters of native grape cultivars of Crimean region; assessment of vegetative and generative potential; calculation of the profitability of cultivation of Crimean native grape cultivars in comparison with the classic cultivars. The result of the research was the selection of genotypes from the group of native cultivars - traits donors and obtaining hybrids of the first generation, which are improved analogs of the Crimean native cultivars.

Keywords: cultivars, grapes, genotype, agrobiological parameters, resistance

1. Introduction

The introduction of varieties and hybrids with high stable yields, high quality products, resistant or tolerant to drought, low temperatures, the most aggressive pathogens and pests, low agricultural background is used in solving problems of resource conservation and environmental protection from destruction and pollution, contributes to the production of environmentally clean products [1–3].

In the process of evolution, native varieties of Crimea developed the properties to grow and produce good quality crops in the conditions of arid climate on poor rocky soils and on soils with a high level of salinity and liming [4, 5]. Changes of climate on our planet lead to the modification of adaptability of plants to the effects

of biotic and abiotic environmental factors [3, 5–7]. In its turn it is expressed in changes of phenology, agrobiology and crop quality parameters [8–11]. The adaptive ability of Crimean native grape cultivars is possible to increase by method of hybridization.

Selection program of grape varieties in the Institute "Magarach" is based on the study of the world gene pool and world trends [8, 12]. In this light, the creation of a new generation of grape varieties - analogues of the Crimean local varieties - highly productive and high quality, carrying genetic adaptability to environmental conditions, while possessing genetically determined signs of resistance to biotic and abiotic factors, is relevant for today. The study of the issue of grape plant resistance, development of practical breeding ways, the study of variability and heredity, the main economic-valuable traits allows us to eventually create and introduce adaptive grape varieties into the industry. New varieties should play an important role in ecologization of viticulture industry.

2. Materials and methods gene pool diversity investigation

The studies were carried out in the Laboratory of Generative and Clonal Selection All-Russian Research Institute of Viticulture and Winemaking "Magarach" on the experimental fields of the Ampelographic Collection Magarach at village of Vilino, Bakhchisarai district, Crimea (44°51′14.8"N 33°38′58.1″E). The area is characterized moderately warm, semi-humid climate: an average annual air temperature of 12,1°C, the sum of active temperatures (above 10 °C) – 3650 - 3680 °C, the number of days with a temperature above 10°C – 197-209, the annual amount of precipitation –380-450 mm. Each cultivar was represented by 10 bushes. Planting scheme of grape plants was 3.0 x 1.5m. Forming –2branch cordon. Grape plants were grafted to the rootstock Kober 5BB. The age of the vineyards is more than 30 years. Agricultural technology system of the ampelographic collection was in accordance with the technological map adopted for each cultivar in the area. The study included native grape varieties of Crimea, related to the direction of use in three groups: wine, table-wine and table.

Assessment of agrobiological and phonological traits was conducted according to the method of Lazarevsky [13] and to the standard OIV method [14]. In short, for each genotype the following trait were recorded: number of latent buds, number of developed shoots, number of fertile shoots, number of inflorescence, number of bunches, average bunch weight (g) and yield per plant (kg). Phytopathological field evaluation was conducted by the examination of untreated plants against a natural infection pressure. In each season, two counts were carried out: the first - after flowering of grapes, the second - at the beginning of grape ripening. The nature and percentage of damage of leaves were scored according to the recommended method [14]. Precisely, on each counting bush up to 30 leaves were evaluated from both sides for signs of infestations. The percentage of affected leaves and the degree of disease development on the leaf were determined using a scale:

- 0—no signs of infestation;
- 1—single, hardly visible spots on leaves (OIV resistance – 9 point);
- 2—up to 10% of leaf surface is affected (OIV resistance – 7 point);
- 3—11-25% of leaf surface is affected (OIV resistance – 5 point);

- 4—26-50% of leaf surface is affected (OIV resistance – 3 point);
- 5—more than 50% of leaf surface is affected (OIV resistance – 1 point).

The study used a laboratory method for testing of frost resistance based on the methodology Chernomorets [15] with some modernization [16].

The data was mathematically processed with the help of statistical software package SPSS Statistics 10.0.

3. Agrobiological and economic assessment of Crimean native grape varieties

The study includes the number of 11 native grape varieties of Crimea and 2control varieties 'Cabernet Sauvignon 'and 'Rkatsiteli'. The study of varieties was carried out with 10 registered bushes in each study in the period of 2010–2012.

The degree of agrobiological characteristics of the variety depends on climatic conditions in the area of cultivation. Taking into consideration the fact that most of the native varieties of Crimea have a functionally female type of flower, weather conditions (in particular, precipitation, strong winds during the blossom period) influenced the processes of inflorescences, formation and berry-filling and, as a result, the mass of bunches and the yield in general.

The beginning of sap flow period was observed from the third decade of March to the first decade of April (**Table 1**).

On average, the beginning of budding was observed from 23 to 26 of April. In 2012 this parameter shifted by 3–4 days in the direction of earlier dates. The earliest bud pushing is the characteristic of the varieties 'Krona', 'Sary Pandas', 'Kok Pandas' and the control variety 'Rkatsiteli'. Blooming in this zone begins after 42–47 days

Variety	Beginning of bud pushing, date	Beginning of blooming, date	Beginning of berries ripening, date	Industrial ripeness, date	Production period, date
Kefesiya	24.04	7.06	8.08	18.09	146
Gevat Kara	26.04	7.06	9.08	16.09	145
Krona	23.04	3.06	6.08	17.09	146
Ekim Kara	24.04	7.06	8.08	18.09	147
Cabernet Sauvignon (c)	25.04	9.06	8.08	19.09	147
Kapselski Belyi	24.04	6.06	7.08	18.09	147
Sary Pandas	22.04	4.06	9.08	15.09	146
Solnechnodolinskii	24.04	5.06	7.08	17.09	143
Kok Pandas	23.04	4.06	5.08	15.09	146
Soldaiya	24.04	6.06	7.08	16.09	145
Shabash	23.04	6.06	6.08	18.09	148
Kokur Belyi	24.04	5.06	4.08	14.09	145
Rkatsiteli (c)	23.04	5.06	6.08	15.09	145

Table 1.
Transit of the main phenological phases in native grape varieties.

from 3 to 9 of June. The group of early flowering includes varieties 'Krona', 'Kok Pandas' and 'Sary Pandas'. The varieties 'Kefesiya', 'Gevat Kara', 'Ekim Kara' and 'Cabernet Sauvignon' (c) are characterized by late flowering. It is necessary to note that 'Solnechnodolinskii' and 'Kokur Belyi' varieties, prone to late budding, entered the flowering phase early. The ripening of berries in studied and control varieties usually occurs after two months, about 59–64 days. The earliest softening of berries is observed in 'Kokur Belyi' variety and occurs on average over the years of study on August, 4; the latest - in the variety 'Sary Pandas'. The earliest coloring of berries begins in 'Krona' variety, latest – 'Gevat Kara'. The onset of industrial ripeness in white varieties is observed the earliest in 'Kokur Belyi' variety (September, 14), the latest in 'Kapselski Belyi' (September, 18). Speaking of the black varieties, the earliest in this group was 'Gevat Kara' (September, 16), the latest was 'Cabernet Sauvignon' (c). On average, the industrial ripeness of the studied varieties practically did not differ and was observed from 14 to 19 of September.

The variety 'Solnechnodolinskii' has the shortest production period of 143 days, and variety 'Shabash' has the longest one of 148 days.

For the period of study, the load of eyes on the bush was distributed as follows: the smallest number was observed in the varieties 'Kefesiya' and 'Ekim Kara', and the biggest - in the variety 'Kokur Belyi' (**Table 2**). The largest percentage of vigorous shoots was observed in the varieties 'Shabash', 'Kapselski Belyi', 'Kokur Belyi', 'Ekim Kara' with share exceeding 90%.

In the variety 'Solnechnodolinskii' the proportion of sterile fruitless shoots does not exceed 50%. Varieties 'Kefesiya', 'Krona', 'Soldaiya', 'Ekim Kara' are characterized by a low number of fruit-bearing shoots – 50-60%. In other native grape

Variety	Bush loading of			Coefficient of	
	eyes, pcs.	shoots, %		fruiting, C1	fertility, C2
		vigorous	fruit-bearing		
Grape varieties with black berry					
Kefesiya	14.0	82.4	53.3	0.66	1.02
Ekim Kara	14.5	91.8	61.2	0.67	1.00
Gevat Kara	19.7	86.8	73.3	0.94	1.11
Krona	20.3	91.8	58.1	0.64	1.01
Cabernet Sauvignon (c)	19.5	82.9	75.7	0.95	1.04
LSD05	2.6	21.9	21.1	0.17	0.03
Grape varieties with white berry					
Kapselski Belyi	24.0	95.2	71.7	0.79	1.05
Solnechnodolinskii	22.3	93.5	46.6	0.51	1.02
Sary Pandas	25.7	88.5	76.7	0.88	1.02
Kok Pandas	25.0	88.4	70.7	0.81	1.01
Kokur Belyi	29.0	94.0	75.2	0.97	1.21
Shabash	24.9	98.7	79.9	0.89	1.10
Soldaiya	22.3	85.2	58.9	0.69	1.00
Rkatsiteli (c)	21.0	89.6	77.4	0.92	1.06
LSD05	1.9	8.9	11.5	0.19	0.02

Table 2.
Agrobiological parameters of grape varieties under study.

varieties the parameters of growth of fruit-bearing shoots do not differ significantly from the control varieties and range from 70 to 80%. The highest fruiting coefficient (C1), approaching the one, had 'Kokur Belyi' and 'Cabernet Sauvignon'. According to the parameter of fruit fertility coefficient (C2) the control variety 'Kokur Belyi' (1.21) significantly differs from the whole group of varieties. Crimean native varieties 'Gevat Kara', 'Shabash' have significant differences in this parameter with the control varieties 'Cabernet Sauvignon' and 'Rkatsiteli'. The highest values of fruiting and fertility coefficients belong to the varieties 'Gevat Kara' (0.94; 1.11), 'Kokur Belyi' (0.97; 1.21). Over the period of study the values of shoot productivity were determined (**Table 3**).

According to the scale of productivity of grape varieties it was established that its level by the parameter of wet raw bunch weight in varieties 'Gevat Kara', 'Kokur Belyi' is characterized as average and do not significantly differ from the control, and in 'Korona' variety, the parameter of shoot productivity is very poor. Low level of shoot productivity in the range from 147 g/shoot to 75.5 g/shoot was noted in all other native varieties under study. The highest crop yield among the black-berried varieties belong to 'Gevat Kara' (62.2 centner/ha) and 'Cabernet Sauvignon' (58.7 centner/ha).

In the group of white-berried varieties the highest yield was observed in 'Kokur Belyi' variety (48.9 centner/ha). By the weight of the bunch, all the studied black-berried varieties are inferior to the control variety 'Cabernet Sauvignon' –176.9 g and variety 'Gevat Kara' –177.9 g. In the group of white-berried varieties the 'Kapselski Belyi', 'Solnechnodolinskii', 'Kokur Belyi' and 'Rkatsiteli' varieties do not differ from the average weight of the bunch. During the onset

Variety	Average weight of the bunch, g	Mass concentration of		Crop yield, centner/ha	Index of productivity, g/shoot
		sugars, Brix	titratable acids, g L^{-1}		
Grape varieties with black berry					
Kefesiya	133.9	22.0	8.4	22.2	88.4
Ekim Kara	112.7	21.0	8.4	22.2	75.5
Gevat Kara	177.9	21.5	8.4	62.2	167.2
Krona	109.7	22.1	7.5	28.9	70.2
Cabernet Sauvignon (c)	176.9	20.6	9.7	58.7	168.1
LSD05	22.8	1.05	1.14	1.3	18.2
Grape varieties with white berry					
Kapselski Belyi	186.2	22.5	6.8	44.4	147.1
Solnechnodolinskii	173.5	22.0	7.5	40.0	88.1
Sary Pandas	125.4	22.5	6.8	28.9	110.4
Kok Pandas	112.9	22.4	7.0	24.4	91.4
Kokur Belyi	185.6	22.1	7.7	48.9	180.0
Shabash	154.3	19.7	10.2	37.8	137.3
Soldaiya	167.0	22.2	7.4	37.8	115.2
Rkatsiteli (c)	187.9	20.0	10.0	44.4	172.9
LSD05	14.5	0.74	0.94	2.6	17.3

Table 3.
Productivity and grape quality of varieties under study.

of technological ripeness, with almost same mass concentration of sugars from 20.6 to 22.1 g L^{-1}, the content of titratable acids significantly decreases from 7.5 to 8.4 g L^{-1} in black varieties compared to the control (9.7 g L^{-1}). In white-berried varieties the sugar content significantly exceeded their concentration in the control variety 'Rkatsiteli' (20.0 Brix), excluding 'Shabash' variety (19.7 Brix). The higher the parameter of the structure (the ratio of the weight of berries to the weight of the stems), the higher the economic value of the variety. To determine this parameter during the study period, the mechanical composition of the crop was studied (**Table 4**).

The smallest proportion of the stem weight in the bunch was observed in the varieties 'Kefesiya' and 'Gevat Kara', the biggest in the varieties 'Ekim Kara' and 'Kok Pandas'. The seeds in the structure of bunch had different quantity and weight, reflected in the percentage of the mechanical composition. It should be noted that Crimean native white-berried grape varieties have low seed weight. According to the parameter of skin weight, following groups may be distinguished: with the lowest value up to 4 percent of the content in the bunch: 'Kapselski Belyi', 'Shabash', 'Soldaiya', 'Kok Pandas'. The highest value of this parameter is observed in the varieties 'Kokur Belyi' and 'Rkatsiteli'. Content of pulp and juice in berries differs by variety: from 82.6 to 91.6%. The highest content of pulp and juice in berries was observed in 'Kapselski Belyi' variety. The highest structural parameter was observed in varieties 'Kefesiya' – 46.7.

Main parameters characterizing the economic value of the variety are: crop yield, cost of production, net income of the product obtained, and level of production profitability. According to the indexed calculation of the above parameters, all native varieties are profitable (**Table 5**).

Due to the low yield and high net cost of the cultivated grapes the varieties 'Ekim Kara' and 'Kefesiya' have a low profitability. The most profitable varieties are 'Gevat Kara' – 273.1%, 'Kokur Belyi' – 144.6%, 'Kapselski Belyi' – 122.0%.

Variety	Weight of				Parameter of structure
	stem, %	seeds, %	skin, %	pulp and juice, %	
Kefesiya	2.1	5.2	7.9	84.8	46.7
Gevat Kara	2.9	5.8	7.0	84.3	33.4
Ekim Kara	4.3	4.6	6.0	85.1	22.3
Krona	3.0	5.1	7.5	84.4	32.2
Cabernet Sauvignon (c)	3.8	5.9	7.7	82.6	25.2
Kapselski Belyi	3.0	2.0	3.4	91.6	31.8
Solnechnodolinskii	3.9	3.3	6.8	86.0	246
Sary Pandas	3.5	3.5	5.1	87.9	27.5
Kok Pandas	4.7	3.2	4.0	88.1	20.2
Soldaiya	3.8	1.8	3.9	90.5	25.2
Kokur Belyi	3.4	2.3	10.0	91.0	28.4
Shabash	3.0	3.6	3.5	89.9	32.3
Rkatsiteli (c)	3.8	6.8	10.9	78.4	25.1

Table 4.
Mechanical composition of bunches of varieties under study.

Variety	Crop yield, centner/ha	Cost of production of 1c, RUB.	Net income of 1c, RUB.	Profitability of production, %
Kefesiya	22.2	2252	748	33.2
Ekim Kara	22.2	2252	748	33.2
Gevat Kara	62.2	804	2196	273.1
Krona	28.9	1730	1270	73.4
Cabernet Sauvignon (c)	60.0	833	1167	140.1
Kapselski Belyi	44.4	1126	1374	122.0
Solnechnodolinskii	40.0	1250	1250	100.0
Sary Pandas	28.9	1730	770	44.5
Kok Pandas	24.4	2049	451	22.0
Kokur Belyi	48.9	1022	1478	144.6
Shabash	37.8	1323	1177	88.9
Soldaiya	37.8	1323	1177	88.9
Rkatsiteli (c)	44.4	1126	674	59.9

Table 5.
Economic effectiveness of cultivation of native grape varieties of Crimea.

4. Variability of crossbreeding of Crimean native grape varieties

Previous studies have determined advisable parameters for assessment of the effectiveness of hybridization of grapes [17–19]. Broadly speaking, the analysis of the effectiveness of hybridization includes an assessment of the crossing ability of the initial forms, risk of loss of a valuable genotype and combination ability, heterosis and transgression [20]. For practical work following evaluation parameters are used:

- setting ability of seeds during self-pollination and cross pollination;
- effectiveness of pollination;
- biological effectiveness of hybridization;
- breeding effectiveness of hybridization.

Setting ability of seeds is estimated as a ratio of the number of seeds to the number of inflorescences taken into consideration [20, 21]. The pollination efficiency expresses the yielding of seeds relative to the theoretically possible number of seeds from all pollinated inflorescences of a particular cross-combination. Biological efficiency of hybridization reflects the efficiency of pollination, vitality and germinating ability of seeds, survival rate of seedlings in a hybrid nursery-garden, the yield of seedlings. Breeding efficiency of hybridization reflects the efficiency of pollination, the vitality and germination of seeds, survival rate of seedlings in a hybrid nursery-garden, the total yield of seedlings and the yield of economically valuable hybrids and may be used as a final assessment of the efficiency of hybridization, but it is somewhat subjective. 142 combinations of crossing of intraspecific and interspecific hybridization performed in the period 2005–2019 were analyzed

Female form	Number of inflorescences	Number of berries formed	Seeds total	Number of seeds per cross-combination	Total of yearlings	Number of seedlings per 1 cross-combination
♀ Aibatly	26	249	325	23.2	227	16.2
♀ Kefesiya	48	2873	4775	227.4	1917	91.3
♀ Krona	29	663	847	60.5	379	27.1
♀ Kok Pandas	90	3688	6093	196.5	1636	52.8
♀ Sary Pandas	91	2468	3605	112.7	1466	45.8
♀ Tashly	32	1176	2566	285.1	947	105.2
♀ Khersonesskii	22	137	151	16.8	65	7.2
♀ Kokur Chernyi	9	300	364	91.0	33	8.3
♀ Misgiuli Kara	11	555	626	156.5	157	39.3
♀ Misket	9	243	295	73.8	104	26.0
Total	367	12352	19647		6931	

Table 6.
The results of hybridization 2005–2018.

(**Table 6**). The study included: as female forms - 10 native varieties of Crimea with a functional female type of flower; as male forms - the pollen of 25 complex interspecific hybrids, 7 varieties of the West European ecological-geographical group and 9 native varieties of the Don were used.

The selection of female forms was carried out on the basis of a complex analysis of prospects of the variety (productivity, crop quality). Since the formation of berries (and setting of seeds) carries the nature of biological features of original female variety and depends on the male form to a small extent [18, 22], it is important to study these parameters in native varieties and distinguish those with the maximum potential for reproduction.

The number of 19647 seeds and 6931 yearlings were obtained as a result of hybridization during pollination of 367 inflorescences. The biggest number of crossings was carried out with the participation of the female parents 'Sary Pandas' and 'Kok Pandas'. At the same time, the maximum number of seeds and hybrid seedlings per one cross-combination was obtained with the participation of the varieties 'Tashly' and 'Kefesiya'. Minimum number of seeds and hybrid seedlings per one combination of crossing was noted in the varieties 'Khersonesskiy' and 'Aibatly'. In combinations involving varieties 'Sary Pandas' and 'Kok Pandas', with the maximum number of inflorescences involved in hybridization, the percentage

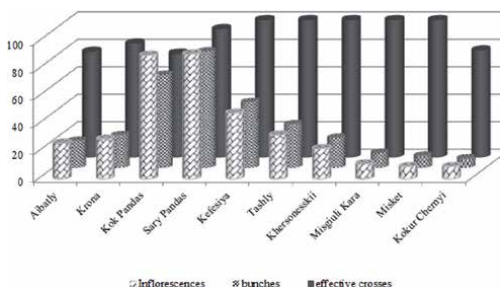

Figure 1.
Crossing efficiency of native varieties of Crimea.

of successful crossings was 93.4 and 75.6% respectively (**Figure 1**). Estimating the percentage of successful crosses, there is a tendency to its decrease with an increase in the number of cross-combinations (Pearson's pair correlation coefficient 0.9604).

Analysis of the results in the context of years showed that the most successful according to the parameters of crossbreeding were 2012 and 2016, and the least favorable were 2015 and 2018 (**Table 7**). Evaluating the variability of the parameter of setting ability of seeds in different years, it was noted that different varieties have high values, as presented in **Figure 2**.

From data presented in **Table 8** it follows that on average over the years of study maximum number of berries and seeds was obtained in cross-combinations involving 'Kok Pandas' variety. In different cross-combinations the female form of 'Kok Pandas' provides the biggest number of berries in one bunch – 62.0 pcs., by the number of fully formed seeds it has average value range - 1.25 pcs., but it still has the smallest fully formed seeds percentage of the total number- 66.7.

Varieties 'Khersonesskii' and 'Aibatly' form the smallest number of berries per one bunch of all studied grapevine cultivars – 5.8 and 8.3 pcs respectively. The number of fully formed seeds per one berry in combination with 'Aibatly' variety is quite high – 1.31 pcs. Combinations involving the varieties 'Kefesiya' and 'Tashly' provide a fairly high number of berries in one bunch – 59.1 and 48.1 pcs, the highest number of fully formed seeds per bunch is 1.59 and 1.77 pcs and the percentage of fully formed seeds is more than 90 of the total.

Parameter	2005	2012	2015	2016	2018	2019	Total
Number of:							
experiments*	8	4	3	6	4	8	33
cross-combinations	32	38	15	25	32	56	198
inflorescences	75	74	63	75	80	138	505
seeds	4311	5121	1303	7486	1428	3816	23465
Setting ability of seeds:							
average	49.6	68.9	21.3	107.1	32.4	43.4	53.8
error of average	15.59	11.41	5.51	22.24	8.65	11.1	12.4
standard deviation	85.38	70.33	21.35	106.64	37.72	38.44	60.0
range of variation	435	272	73	319	161	136	233

**- number of experiments is equal to the number of female varieties involved in hybridization.*

Table 7.
Setting ability of seeds in different years of study.

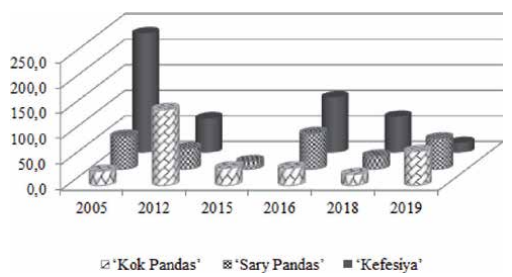

Figure 2.
Changes in setting ability of seeds of 'Sary Pandas', 'Kok Pandas' and 'Kefesiya' grape varieties

Female form	Number of berries		Number of seeds			Fully formed seeds, % of the total number
	total	per one bunch	total	fully formed	fully formed per one berry	
♀ Aibatly	249	8,3	325	313	1.31	83.0
♀ Kefesiya	2873	59.1	4775	4486	1.59	95.6
♀ Krona	663	23.9	847	779	1.19	84.8
♀ Кок Pandas	3688	62.0	6093	4421	1.15	66.7
♀ Sary Pandas	2468	26.8	3605	3130	1.25	83.5
♀ Tashly	1176	48.1	2566	2408	1.77	93.7
♀ Khersonesskii	137	5.8	151	122	0.79	75.6
♀ Kokur Chernyi	300	22.1	364	347	0.96	84.4
♀ Misgiuli Kara	555	40.9	626	564	0.93	80.6
♀ Misket	243	21.0	295	276	0.80	72.0
Average value	1235	32	1965	1685	1.00	82.0
Coefficient of variation	104.7	62.30	110.43	104.93	27.62	10.88
Percentage error of average	33.1	19.70	34.92	33.18	8.73	3.44
Confidence range (+/–)	801.5	12.28	1344.7	1095.6	0.20	5.53

Table 8.
Viability of hybrid seeds.

Germination of seeds depends on hereditary strength, consisting in the fact that the necessary tissues and organs are formed and matured to ensure germination in appropriate conditions. Obtaining of seeds of low viability is determined by the female genotype long before the pollination [18, 22, 23].

Considering the parameters of seed germination with the participation of various native varieties, high data variability is noted (**Table 9**).

So in the cross-combinations with the participation of 'Aibatly' and 'Khersonesskii' varieties, the average number of seedlings per one cross-combination has a very low level - 7.2-16.2 pcs. Moreover, the seedlings obtained from fully formed seeds amount a very high percentage - more than 60.

In the total selection of the studied varieties, the female form 'Tashly' stands out, as it provides in hybridization the maximum number of seedlings per 1 combination of crossing, more than 100 pcs. The average percentage of seedlings obtained from full seeds is very low – 30.7 and the maximum level is 48%. The maximum variability of parameters of seed viability was noted in varieties 'Kok Pandas' and 'Sary Pandas'. Further, during the analysis of data for practical determination of the effectiveness of hybridization, we dwelt on the definition of 3 complex parameters: seeds setting; pollination efficiency; biological effectiveness of hybridization. **Table 10** presents these parameters in numerical terms, specific for the group of varieties under study and showing the range of variation of these parameters.

Analyzing the clustering results of the studied group of autochthonous varieties (**Figure 3**), we see that the varieties divided into 2 separate clusters: I – group,

Female form	Total yearlings	Number of seedlings per one cross-combination	Seedlings, % of fully formed seeds	
			average (x)	limits (x max – x min)
♀ Aibatly	227	16.2	64.0	16.6–100.0
♀ Kefesiya	1917	91.3	51.6	9.1–70.0
♀ Krona	379	27.1	51.2	28.7–88.5
♀ Кок Pandas	1636	52.8	42.3	2.6–100.0
♀ Sary Pandas	1466	45.8	48.0	1.5–93.8
♀ Tashly	947	105.2	30.7	4.2–48.0
♀ Khersonesskii	65	7.2	60.8	40.0–100.0
♀ Kokur Chernyi	33	8.3	21.9	5.6–38.1
♀ Misgiuli Kara	157	39.3	30.9	23.7–36.2
♀ Misket	104	26.0	48.3	36.1–58.8

Table 9.
Variability of parameter "germination of seeds".

Female form	Setting of seeds	Pollination efficiency	Biological effectiveness of hybridization
♀ Aibatly	14.4 ± 7.76 40.5	0.0002 0.0005	0.0001 0.0003
♀ Kefesiya	101.1 ± 45.10 436.0	0.0009 0.003	0.0004 0.001
♀ Krona	32.1 ± 14.01 76.5	0.0005 0.001	0.0003 0.0006
♀ Кок Pandas	97.9 ± 38.07 316.2	0.002 0.007	0.0007 0.003
♀ Sary Pandas	39.0 ± 13.87 141.0	0.0009 0.003	0.0004 0.002
♀ Tashly	106.0 ± 63.49 321.3	0.0006 0.002	0.0002 0.001
♀ Khersonesskii	6.4 ± 3.37 14.3	0.0004 0.001	0.0002 0.001
♀ Kokur Chernyi	35.2 ± 13.96 79.0	0.0006 0.0001	0.0001 0.00013
♀ Misgiuli Kara	44.7 ± 31.12 97.8	0.001 0.003	0.0004 0.001
♀ Misket	25.5 ± 12.84 91.0	0.0004 0.001	0.0002 0.0004
Average of variants of crossing	59.7 ± 13.82 436.0	0.0009 0.007	0.0004 0.003

In denominator indicates the range of variation of the value.

Table 10.
Effectiveness of hybridization of native varieties.

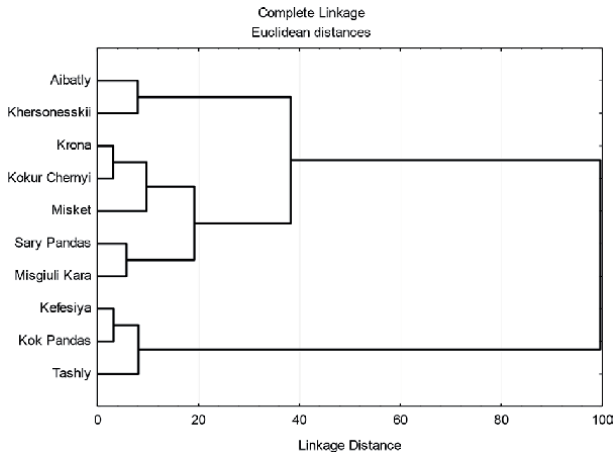

Figure 3.
Multifactorial hierarchic classification of genotypes under study by the effectiveness of their hybridization.

consisting of 3 varieties: 'Kok Pandas', 'Kefesiya' and separate variety 'Tashly'; II – group, including 7 varieties – divided into 2 big subclusters: a) varieties 'Aibatly' и 'Khersonesskii'; b) 'Krona', 'Kokur Chernyi' and separate varieties 'Sary Pandas', 'Misgiuli Kara' and 'Misket'.

A group of varieties including 'Kefesiya', 'Kok Pandas' and 'Tashly', was selected in the analysis and demonstrates high hybridization efficiency in intraspecific crossing and in crossing with complex interspecific hybrids. Varieties 'Sary Pandas' and 'Misgiuli Kara' are distinguished by low setting ability of seeds, however, the biological effectiveness of hybridization remains at the level of group 1. Thus, we can confirm that female parent varieties 'Sary Pandas' and 'Misgiuli Kara' are specific in issues of crossing ability and viability of hybrid seeds.

5. Features of breeding of grape genotypes resistant to oidium in crossing of Crimean native varieties with complex interspecific hybrids

Analysis of the laws of inheritance of resistance to oidium in hybrid progeny makes it possible to carry out scientific selection of initial forms for immunoselection programs realization. These objective laws are established on the basis of the study on a fixed infection background of representational material of hybrid populations obtained in the process of crossing of various parental forms with resistance to the pathogen. In different cross-combinations the variability of feature of oidium resistance was revealed.

A significant number of highly susceptible to oidium seedlings, up to 7%, was obtained in crossings with participation of varieties 'Sary Pandas' and 'Misgiuli Kara'. The biggest percentage rate of highly resistant seedlings (9 points) was recorded in the combination of 'Khersonesskii' x 'JS 26–205' (22%). Crossings of 'Kok Pandas' x 'Tsitronnyi Magaracha' (4.5 points), 'Kokur Chernyi' x 'Ifigenia' (4.3 points), 'Misket' x 'Ifigenia' (4.3 points), 'Muscat Jim' x 'Kokur Belyi' (4.5 points) mostly followed to the formation of medium-resistant to oidium forms. It should be noted that the average score of resistance to oidium in all populations was higher than in the initial Crimean native varieties.

The breeding value shows the possibility of distinction of highly-resistant, resistant and medium-resistant to oidium plants in hybrid population in the contrast to the sensitive Crimean native varieties. It was determined as the

Cross-combination		Breeding value, %	Coefficient of variation, %	Dominance degree, %	Heterosis hypothetic, %
Magarach No. 31–77-10'	**Gevat Kara**	**3.5**	**32.7**	**2.19**	**36.5**
Kok Pandas	Spartanets Magaracha	0.0	33.1	0.76	30.3
Muscat Jim	Shabash	10.7	40.0	0.75	30.0
Kefesiya	Spartanets Magaracha	2.0	36.8	0.60	24.0
Sary Pandas	Spartanets Magaracha	7.8	37.1	0.38	15.1
Misgiuli Kara	Spartanets Magaracha	7.4	36.6	0.07	1.9
Kokur Chernyi	Ifigenia	0.0	37.2	−0.26	−6.5
Misket	Ifigenia	0.0	22.8	−0.32	−8.0
Kok Pandas	Tsitronnyi Magaracha	0.0	20.3	−0.46	−11.5
Khersonesskii	JS 26–205	0.0	21.9	−0.78	−13.0
Tashly	Krymchanin	0.0	16.8	−2.00	−50.0

Table 11.
Breeding characteristics of hybrid populations by oidium resistance.

percentage of seedlings in populations with 5, 7 and 9 points of oidium resistance. Cross-combinations (**Table 11**) involving complex interspecific hybrids of varieties 'Muscat Jim', 'Spartanets Magaracha' and 'Magarach No. 31-77-10' had the highest breeding value. The most effective was the combination of 'Muscat Jim' x 'Shabash' with the yield of resistant and highly-resistant seedlings 10.7 percent. Degree of the dominance reflects the contribution of parent components to the variability of the trait. Negative values of the degree of dominance show that the deviation of the traits of resistance to oidium goes to the direction of more susceptible parental form. The degree of dominance shows that in 'Tashly' x 'Krymchanin' there is a hybrid depression, in the population of 'Kokur Chernyi' x 'Ifigeniya', 'Misket' x 'Ifigenia', 'Kok Pandas' x 'Tsitronnyi Magaracha', 'Khersonesskii' x 'JS 26–205' - there is a deviation to a more susceptible parent. In populations 'Kok Pandas' x 'Spartanets Magaracha', 'Muscat Jim' x 'Shabash', 'Kefesiya' x 'Spartanets Magaracha', 'Sary Pandas' x 'Spartanets Magaracha' - there is a slight dominance of more stable parent. Only in one population 'Magarach No. 31–77-10' x 'Gevat Kara' (2.19%) there was a deviation to a more stable parental form.

In populations with the participation of Crimean native varieties 'Misgiuli Kara', 'Sary Pandas', 'Kefesiya', 'Shabash', 'Kok Pandas', 'Gevat Kara' and 'Magarach No. 31-77-10' x 'Gevat Kara', hypothetic heterosis from 1.9 to 36.5 percent was noted. The transgressive recombinants were not observed in the studied combinations.

One of the main parameters characterizing the genetic potential of parental forms is the hereditability of breeding traits. The effectiveness of breeding selection in the studied populations is characterized by the parameter of hereditability of the trait, which is determined by the method of dispersion analysis of single-factor complexes. To calculate the hereditability indices, 13 single-factor complexes, including from 2 to 6 cross-combinations, were organized (**Table 12**). The lowest

Variety	Number of seedlings in the complex, pcs	Average score of resistance to oidium in the complex	Parameter of the power of influence of the variety	Parameter of reliability of the influence of the variety	Standard values of the criterion of Fisher
Female forms					
Sary Pandas	269	3.8	0.0	6.5	{1.6–2.0 – 2.6}
Muscat Jim	105	4.2	0.1	4.3	{2.0–2.6 -3.4}
Kok Pandas	81	3.9	0.0	3.1	{1.7–2.0 – 2.7}
Magarach No. 31–77-10	131	4.1	0.1	2.5	{1.6–2.0 – 2.6}
Misgiuli Kara	139	3.7	0.0	1.6	{1.6–2.0 – 2.6}
Kefesiya	161	3.7	0.0	1.6	{1.6–2.0 – 2.6}
Kokur Chernyi	53	4.2	0.0	0.1	{2.0–2.7 – 3.5}
Male forms					
Spartanets Magaracha	249	4.4	0.1	7.7	{1.6–2.0 – 2.6}
Ifigenia	369	3.9	0.0	6.1	{1.6–2.0 – 2.6}
Tsitronnyi Magaracha	129	4.1	0.1	5.3	{1.7–2.0 –2.6}
Gevat Kara	111	3.8	0.0	5.1	{1.7–2.0 – 2.7}
Shabash	68	3.9	0.0	5.0	{1.7–2.0 – 2.7}
Kokur Belyi	57	3.7	0.0	0.1	{1.7–2.0 – 2.7}

Table 12.
Dispersive parameter of inheritance of resistance to oidium.

average score of 3.7 by the complex trait of resistance to oidium of Crimean native female forms possessed combinations of 'Kefesiya' and 'Misgiuli Kara' varieties, the highest - 'Kokur Chernyi' variety (4.2%), but the data presented for the last variety was not reliable (0.1). Inaccuracy did not indicate the absence of the influence of parents on genetic diversity of the progeny, but was explained by the limited number of seedlings in populations and small number of cross-combinations in some single-factor complexes. Average values of the remaining female forms did not exceed 4 points, and ranged in 3.8 points for 'Sary Pandas' variety and 3.9 points for 'Kok Pandas'. In crossbreeding complexes with Crimean natives, where interspecific varieties 'Muscat Jim' and 'Magarach No. 31-77-10' were used as female forms, the resistance to oidium was 4.2 and 4.1 points respectively. The highest resistance among the complexes of male forms was observed in the variety 'Spartanets Magaracha'.

For female varieties strength of the influence (0.1) of interspecific varieties 'Muscat Jim' and 'Magarach No. 31-77-10' on the inheritance of resistance to oidium of the progeny in crossing with Crimean natives is reliably confirmed. Values of this parameter, 4.3 and 2.5, indicate that usage of these varieties as female forms in crossing with Crimean native varieties will make it possible to obtain stable seedlings in F1 depending on the specific combining ability of the parental components. The dispersion complexes of the Crimean natives 'Sary Pandas', 'Kok Pandas', 'Migiuli Kara', 'Kefesiya' and 'Kokur Chernyi' are characterized by zero influence on the progeny's resistance to oidium, as confirmed by parameters of reliability. The use of these varieties as parental forms with various donors of resistance to oidium will not allow to obtain a significant number of resistant genotypes in F1.

It is established reliably that high proportion of genotypically determined inheritance of the trait of resistance to oidium is observed in crossing with male forms of interspecific origin 'Spartanets Magaracha', 'Tsitronnyi Magaracha'. In other words, these donors of oidium resistance, regardless the stability of another parental component, provide a high yield of oidium resistant forms in hybrid populations. Local varieties of Crimea 'Gevat Kara', 'Shabash' and 'Kokur Belyi' do not affect the oidium resistance of their progeny.

6. Frost resistance of Crimean native grape varieties and their hybrids

Determination of frost-resistant native varieties of Crimea to identify sources of relative frost resistance and selection to the elite of the most frost-resistant genotypes obtained by crossing of native varieties of Crimea and hybrid varieties of complex interspecific origin is a promising direction of breeding work. The research objectives included: assessment of frost resistance of native varieties of Crimea by laboratory methods; selection to the elite the most frost-resistant genotypes obtained as a result of hybridization of Crimean native grape varieties with the complex interspecific hybrids.

As a result of the study, the frost resistance of 15 original forms, local varieties of Crimea, was tested using the laboratory method of assessment (**Figure 4**). The least frost resistance among the studied parental forms, local varieties of Crimea, showed the varieties 'Shabash', 'Soldaiya' and 'Solnechnodolinskii'. The best frost resistance to minus 24°C among the analyzed local varieties of Crimea was shown by the varieties 'Khersonesskii' and 'Kapselski'.

The results of our researches correspond to the results of assessment of the reaction of 84 Crimean native grape varieties of the ampelographic collection of the Magarach Institute on the influence of extreme winter temperatures of 2006 (−22.5 °C) obtained by the field method. An assessment of the preservation of the main and base buds, as well as the analysis of regenerative ability of the bushes, allows us to divide the studied varieties by frost resistance into three groups:

- the first group of non-resistant grape varieties; loss of 100% of main buds; loss of 95–100% of base buds; includes 57 varieties: 'Kandavasta', 'Kozskiy Stolovyi;' Nasurla', 'Shabash' and others; recovery of bushes of the remaining

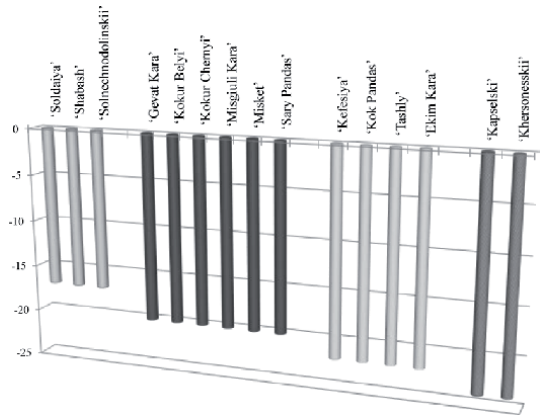

Figure 4.
Differentiation of native grape varieties of the Crimea by resistance to frost.

varieties was carried out with the help of base buds on fruit canes and sleeping buds of old wood of arms of the trunk and bush head;

- the second group of varieties: preservation of main buds in these varieties was 0%, base buds - 1-9%; 5% of budded shoots on fruit canes; 5% - 50% of budded shoots with the help of sleeping buds of perennial wood; consists of 20 varieties: 'Kanagyn Izium', 'Kefesiya', 'Kok Pandas', 'Solnechnaya Dolina 71/7', 'Firskii Ranniiy', 'Shira Izium' and others.

- the third group of relatively resistant grape varieties: preservation of main buds in these varieties was 0–7%, base buds - 3-25%; 25–50% of budded shoots on fruit canes; 5–50% of budded shoots with a help of sleeping buds of perennial wood; these are the varieties 'Chivsiz Sary', 'Dere Izium', 'Solnechnaya Dolina 41', 'Biyas Aibatly', 'Kutlakskii Chernyi', 'Kapselski' and 'Khersonesskii'.

All local varieties of Crimea belong to different ecological and geographical groups by their origin [5, 9]. Varieties 'Misgiuli Kara', 'Sary Pandas', 'Shabash' belong to the eastern ecological-geographical group - convar. *orientalis* Negr.; varieties 'Gevat Kara', 'Kokur Belyi', 'Misket', 'Tashly', 'Khersonesskii' belong to the ecological-geographical group of varieties of Black Sea Basin - convar. *pontica* Negr.; variety 'Kok Pandas' belongs to the west-european ecological-geographical group - convar. ossidentalis Negr. Separating the studied local varieties of Crimea into the groups of frost resistance, it should be noted that genotypes of convar. *pontica* Negr. and convar. ossidentalis Negr. possess high and average resistance to low temperatures, and varieties of the ecological-geographical group of convar. *orientalis* Negr. are classified as low frost-resistant and non-frost-resistant. In general, the data of resistance to low temperatures in various ecological-geographical groups correspond to the available literature sources.

During an agrobiological study in the period 2012–2015 the numbers of 21 elite forms were selected from 296 promising seedlings of the Crimean native varieties crossed with the complex interspecific hybrids. The yielded vine passed similar to the above method of laboratory freezing tests. It is established that the buds of eight elite seedlings hold reduction of temperature to minus 22°C (**Figure 5**).

After freezing through at minus 24°C hardwood cuttings of the following populations were capable to green shoots formation: 'Magarach No. 7-08-15-3', 'Magarach No. 11-08-17-2', 'Magarach No. 10-08-16-1', 'Magarach No. 10-08-8-3', 'Magarach No. 11-08-15-2', 'Magarach No. 11-08-13-3', 'Magarach No. 10-08-14-2', 'Magarach No. 10-08-17-2', 'Magarach No. 4-08-17-3', 'Magarach No. 5-08-8-4', 'Magarach No. 4-08-3-3'.

Freezing through at temperature of minus 26 °C of hardwood cuttings of elite form 'Magarach No. 8-08-8-4' ('Kok Pandas' x 'Zeibel No. 6357') did not follow to the damage of buds, and gave normal shoots after the exit of dormant state. The forms selected to the elite in each population have different frost resistance. Such difference is observed in the population of 'Sary Pandas' x 'Tsitronnyi Magaracha' in the form 'Magarach No. 7-08-7-3': frost resistance is minus 22°C, and in the form 'Magarach No. 7-08-15-3' it reaches minus 24°C. Similar situation was revealed in the population 'Kefesiya' x 'Ifigenia', where the elite form 'Magarach No. 10-08-8-2' is characterized by frost resistance of minus 22°C, and 'Magarach No. 10-08-8-3' - of minus 24°C. In the population 'Misket' x 'JS 26205', the form 'Magarach No. 4-08-17-4' withstands freezing through to minus 22°C, and the forms 'Magarach No. 4-08-17-3' and 'Magarach No. 5-08-8-4' - to minus 24°C. Moreover, almost all elite forms, in contrast to the initial Crimean native varieties in populations, are characterized by frost resistance higher by 2 °C.

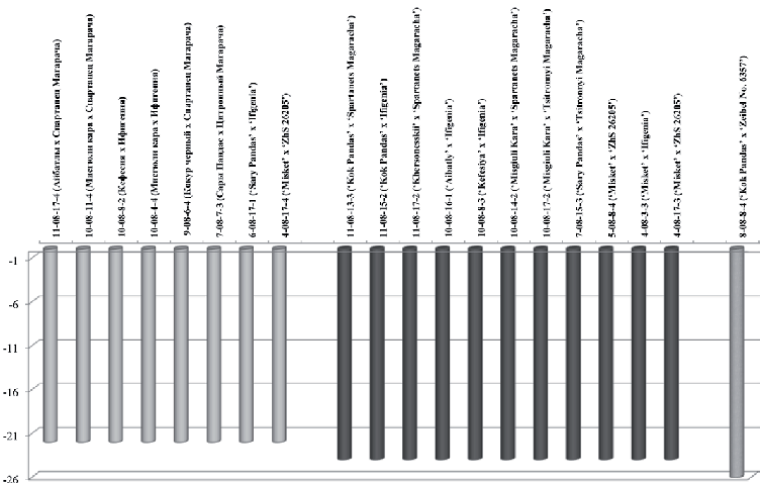

Figure 5.
Resistance to frost in hybrids of native varieties of Crimea.

7. Agrobiological specificity of breeding forms - analogues of Crimean native grape varieties

The production compatible in the internal and international market is a national patrimony; in viticulture and winemaking this is the production made of unique native grape varieties. The introduction of new grape varieties, analogues of the Crimean autochthones, having a genetically determined association of qualitative and quantitative traits in combination with resistance to environmental stress factors, will increase the economic efficiency of viticulture and winemaking industry. We studied on a combination of parameters 10 promising black-berried forms obtained in crossing of native grape varieties of Crimea with complex interspecific hybrids. Analyzing the production period of the hybrid forms highlighted as elite in comparison with the control variety 'Kefesiya', the dates of onset of phenological phases should be specially indicated (**Table 13**).

On average, in 2012–2015, the study of buds pushing in the researched hybrid forms began on April, 23. The coefficient of variation of this characteristic had an insignificant (10%) range of values of the trait in statistical population. The established standard deviation of 2,3 days allowed us to determine the varietal peculiarity of an earlier bud pushing for 3 days (April, 21) in the elite forms 'Magarach №10–08–8-2' and 'Magarach №10–08–8-3' compared to their initial form 'Kefesiya', studied as a control (April, 24). Blooming of the studied forms begins on average on June, 7 and coincides with the control variety. Range of the dates of blooming from June, 5 to June, 10 is determined by a coefficient of variation of 24.5%. Moreover, in 4 elite forms ('Magarach № 5-08-8-4', 'Magarach № 10-08-4-4', 'Magarach № 10-08-17-2', 'Magarach № 11-08-9-2') there is a deviation towards a later onset of blooming with an excess of the standard deviation (1.8 days) in comparison with the control. Totally the onset of blooming in the studied genotypes does not carry the character of significant difference. The beginning of the ripening period of berries in the studied forms was observed on average on August, 6. A significant difference between the genotypes was revealed at the stage of technological ripeness with the content of sugars in berries 21–22 Brix. The range of variability of onset of the technological ripeness (September, 16) in the average exceeded 33% and reached 39.4, which indicated the general dissimilarity of the whole in a trait.

Hybrid form, Magarach No.	Cross-combination		Onset of bud pushing, date	Onset of blooming, date	Onset of ripening of berries, date	Industrial ripeness, date	Productive period, days
	♀	♂					
5–08–8-4	Misket	JS 26205	25.04	9.06	7.07	9.09	140
10–08–8-2	Kefesiya	Ifigenia	21.04	6.06	5.07	9.09	143
10–08–8-3	Kefesiya	Ifigenia	21.04	6.06	5.07	9.09	143
10–08–4-4	Misgiuli Kara	Ifigenia	26.04	10.06	9.07	15.09	145
4–08–3-3	Misket	Ifigenia	20.04	5.06	3.07	9.09	145
9–08–6-4	Kokur Chernyi	Spartanets Magaracha	22.04	7.06	5.07	15.09	149
10–08–17-2	Misgiuli Kara	Tsitronnyi Magaracha	26.04	10.06	8.07	23.09	153
11–08–9-2	Khersonesskii	Spartanets Magaracha	25.04	9.06	8.07	23.09	155
10–08–14–3	Misgiuli Kara	Spartanets Magaracha	21.04	6.06	5.07	23.09	157
10–08–11-4	Misgiuli Kara	Spartanets Magaracha	21.04	6.06	5.07	23.09	157
Kefesiya (c)			24.04	7.06	8.07	18.09	146
$\bar{x}$			23.04	7.06	6.08	16.09	148
σ			2.30	1.80	1.89	6.31	6.09
V, %			10.0	24.5	30.5	39.4	4.1

Table 13.
Phenology of hybrids of native grape varieties of the Crimea.

The established biological variability of this trait, according to the existing gradation of the OIV scale, made it possible to distribute the studied genotypes by terms of ripening. Forms 'Magarach No.5–08–8-4', 'Magarach No.10–08–8-2', 'Magarach No.10–08–8-3', 'Magarach No.10–08–4-4', 'Magarach No.4–08–3-3', 'Magarach No.9–08–6-4' refer to varieties of average term of ripeness - 4 points – September, 01–15, and forms 'Magarach No.10–08–17-2', 'Magarach No.11–08–9-2', 'Magarach No.10–08–14–3', 'Magarach No.10–08–11-4' and control variety 'Kefesiya' – to varieties of average-late term of ripeness - 5 points – September, 16–30. To determine the biological productivity of the studied promising forms, it is necessary to consider their bearing potential (**Table 14**).

Among the studied genotypes the least development of shoots was observed in forms 'Magarach No. 10-08-8-2', 'Magarach No. 9-08-6-4', 'Magarach No. 10-08-11-3'. In other forms this trait did not have significant differences compared to the control and was in the range 62.5–86.4%.

Fruit-bearing coefficient is one of the main parameters determining the potential productivity of genotypes. Among the forms under study, a very low fruit-bearing coefficient was noted in the genotypes 'Magarach No.10–08–8-2', 'Magarach No.9–08–6-4' and 'Magarach No.4–08–4-3'. Elite form 'Magarach No.10–08–17-2' had fruit-bearing coefficient (1.1) much higher than the control (0.66). Productiveness of the shoot in the wet raw bunch weight (g/shoot) should be considered as a resulting parameter of crop efficiency of variety. The inheritance of the forms under study with distinct direction of the trait value downwards was observed taking into account the varietal peculiarity of Crimean native wine grape cultivars and initial low productivity. Four studied forms were characterized by shoot productivity at the level of the control variety 'Kefesiya'.

The adjusted varietal specificity of the potential productivity of ten promising forms allowed to select four hybrid forms to the elite – 'Magarach No.5–08–8-4', 'Magarach No.10–08–4-4', 'Magarach No.10–08–8-3', 'Magarach No.10–08–14–3'.

Qualitative characteristics of promising forms were studied together with the determination of the productive period and fruit-bearing potential (**Table 15**). On average, among the studied forms the juice output was 58.2%. According to

Hybrid form, Magarach No.	Shoot formation per bush, %		Coefficient		Productivity of the shoot, g/shoot
	developed	fruit-bearing	C1	C2	
10–08–8-2	50.0	22.2	0.22	1.00	42.2
9–08–6-4	46.2	33.3	0.34	1.00	57.7
10–08–11-3	48.1	38.5	0.60	1.20	62.2
4–08–4-3	63.2	29.2	0.31	1.10	67.2
10–08–17-2	66.1	56.4	1.10	1.90	69.8
11–08–9-2	86.4	31.6	0.66	1.00	70.3
10–08–14–3	62.5	46.7	0.57	1.00	75.3
10–08–4-4	70.3	50.0	0.58	1.15	84.7
5–08–8-4	66.7	37.5	0.50	1.33	85.0
10–08–8-3	75.7	44.9	0.50	1.17	86.4
Kefesiya (c)	72.4	55.1	0.66	1.02	88.4
LSD05	15.9	11.1	0.17	0.03	17.2

Table 14.
Crop productivity of hybrids of native grape varieties of the Crimea.

Elite form, Magarach No.	Stem weight, %	Seeds weight, %	Skin and pulp weight, %	Juice output, %	Parameter of structure
5–08–8-4	3.5	8.0	37.1	51.4	18.6
10–08–4-4	2.7	8.9	29.0	59.4	43.0
10–08–8-3	3.9	5.9	29.8	60.2	25.0
10–08–14–3	1.5	11.1	34.5	52.9	67.5
Kefesiya (c)	2.1	5.2	32.4	62.4	46.7
$\bar{x}$	3.0	7.3	30.2	58.2	37.2
σ	1.1	2.5	6.6	8.1	18.7
V,%	35.6	33.9	21.8	14.0	50.3

Table 15.
Mechanical composition of the bunch of elite form.

the gradation of the OIV scale, the studied genotypes 'Magarach No.5–08–8-4', 'Magarach No. 10-08-14-3' belong to the group of varieties with the low output of juice, and the elite forms 'Magarach No. 10-08-4-4', 'Magarach No. 10-08-8-3' and the control variety 'Kefesiya' - to the group of varieties with an average juice output. Form 'Magarach No. 10–08–8-3' in terms of the average weight of the bunch was quite different from initial form 'Kefesiya'. Such a variety was explained by the different type of flower: female in the variety 'Kefesiya' and androgenous in the studied elite form 'Magarach No. 10-08-8-3'. The output yield was recalculated per 1 ha depending on the average yield per bush in elite forms. Records determining the cropping potential of the studied genotypes were obtained. Form 'Magarach No. 10–08–14–3' was characterized by a very low productivity (21.9 center/ha), form 'Magarach No. 5-08-8-4' (45.2 center /ha) did not significantly differ from the control (48.0 center /ha). There was no difference between productivity of elite forms 'Magarach No.10–08–4-4' (53.2 center /ha) and 'Magarach No.10–08–8-3' (55.7 center /ha), but essential increase in crop yield of these genotypes compared to the control variety 'Kefesiya' was revealed.

We have obtained data that determine the potential juice yield per hectare. It allowed us to recommend the elite form for production tests. The highest value of the parameter of juice output per hectare (336.4) among the studied genotypes was noted in form 'Magarach No. 10-08-8-3' ('Kefesiya' x 'Ifigenia') (**Table 16**).

As a general matter, the obtained data of the productive period, potential crop efficiency, mechanical composition and yielding capacity of the studied gene

Elite form, Magarach No.	Bunch weight, g	Yield, kg/bush	Crop productivity, center /ha	Juice output, dL/ha
5–08–8-4	170	1.356	45.2	232.3
10–08–4-4	146	1.595	53.2	315.8
10–08–8-3	173	1.670	55.7	336.4
10–08–14–3	108	0.659	21.9	116.2
Kefesiya (c)	162	1.414	48.0	299.5
LSD05	9.1	0.07	3.7	17.2

Table 16.
Crop productivity of elite forms.

pool, was united to choose and highlight two elite forms 'Magarach No. 10-08-4-4' ('Misgiuli Kara' x 'Ifigenia') and 'Magarach No. 10-08-8-3' ('Kefesiya' x 'Ifigenia').

8. Conclusions

In the process of studying the biology of local grape varieties of the Crimean region investigated the possibility of their use in breeding to obtain more adaptive grape varieties that can be competitive in the viticulture and winemaking market. The grape plant in more than 2000 years of culture has shown in itself an exceptionally high adaptive capacity to stress factors. Nevertheless, thanks to introgression of genes of resistance to drought, low temperatures, and pathogens, we are able to manage the genetic diversity of the crop and create a wide range of new grape varieties.

Thus, we can state: according to the main economic parameters, the most profitable for cultivation without irrigation in the eastern South Coast zone of viticulture of Crimea among the native grape varieties are 'Gevat Kara', 'Kokur Belyi' and 'Kapselski Belyi'. A group of varieties including 'Kefesiya', 'Kok Pandas' and 'Tashly', was selected in the analysis and demonstrates high hybridization efficiency in intraspecific crossing and in crossing with complex interspecific hybrids. Hybridological analysis of the progeny in F1 showed that the average index of resistance to oidium depends on the genetic characteristics of the parent components. Hybridological analysis showed that the most resistant progeny developed in the crossing of 'Khersonesskii' × 'JS 26–205' (6.8 points). It is established that a high degree of genotypically determined inheritance of the trait of resistance to oidium is observed in crossings with the participation of female forms of interspecific origin – 'Magarach No. 31-77-10', 'Muscat Jim' and male forms– 'Spartanets Magaracha' and 'Tsitronnyi Magaracha'. The forms selected to the elite in each population have different frost resistance. Such difference is observed in the population of 'Sary Pandas' x 'Tsitronnyi Magaracha' in the form 'Magarach No. 7-08-7-3': frost resistance is minus 22 °C, and in the form 'Magarach No. 7-08-15-3' it reaches minus 24°C. Almost all elite forms, in contrast to the initial Crimean native varieties in populations, are characterized by frost resistance higher by 2°C. Four genotypes 5–08–8-4 ('Misket' x 'JS 26205'), 10–08–4-4 ('Misgiuli Kara' x 'Ifigenia'), 10–08–8-3 ('Kefesiya' x 'Ifigenia') and 10–08–14–3 ('Misgiuli Kara' x 'Spartanets Magaracha') were selected from the group of native grape varieties - donors of traits and obtaining hybrids of the first generation, which are improved analogues of the native grape varieties of Crimea.

Conflict of interest

The authors declare no conflict of interest.

Author details

Svetlana Levchenko*, Irina Vasylyk, Vladimir Volynkin, Vladimir Likhovskoy and Alla Polulyakh
All-Russian National Research Institute of Viticulture and Winemaking "Magarach", Yalta, Russian Federation

*Address all correspondence to: svelevchenko@rambler.ru

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Chapter 6

Romanian Organic and Conventional Red Grapes Vineyards as Potential Sources of High Value-Added Products, in a Circular Economy Approach

Cristina Mihaela Nicolescu, Marius Bumbac, Cristiana Radulescu, Lavinia Claudia Buruleanu, Radu Lucian Olteanu, Laura Monica Gorghiu, Gabriela Teodorescu and Carmen Georgeta Holban

Abstract

The use of natural ingredients with active functions has been intensively studied in the last years, as a consequence to consumer preferences for organic products. Application of circular economy principles determined a significant research activity in the viticulture field. The use or re-use of vines parts for so-called nutraceuticals or other consumer-goods applications, are basically centered on their phytochemical and microbiological characterization. Eurostat updates ranks Romania fifth among the EU member states, with a total area under vines of 183,717 hectares. Characterization of four *Vitis vinifera L.* varieties, out of which one pure Romanian variety (Feteasca Neagra), cultivated in organic and conventional vineyards, together with pedoclimatic conditions have been provided. Data on phytochemical parameters and antimicrobial activity of extracts obtained from different anatomic parts of grapes were included. Analytical protocols and techniques applied were presented, together with data and results interpretation. Several chemometric algorithms have been used as complementary tools for interpretation of the instrumental analytical data.

Keywords: organic/conventional vineyards, antioxidant activity, polyphenols, flavonoids, spectroscopy, antimicrobial activity, chemometrics

1. Introduction

Sustainability was defined by the United Nations far in 1987 as "the development that meets the needs of the present without compromising the ability of future generations to meet their own needs" [1, 2].

A constant presence in the state-of-the-art scientific literature consists of studies aiming at identifying and testing various possibilities to re-use various by-products

generated in the field of vine crops and wine industry. A positive economic impact, together with a positive social and environmental impacts on long term are aimed, actions focusing on obtaining high-value added products, and on thoroughly defining the benefits of organic over conventional viticulture [3–8].

Waste from economical activities related to vine cultures may be solid or liquid. Wastes may be generated in different technological phases of wine industry, and in other grape-based foods or beverages. Also, a significant amount of waste comes from the cultivation of vines itself. Solid waste materials may be grape stalks, grape seeds, grape pomace and others. Grape stalks are the major byproduct of the vineyards, and may be an important source of cellulose, lignin, sodium (Na) and potassium (K) [9], while grape pomace is the major waste from wine industries [10]. Grape pomace consists of skin residues, pulp remains, stalks, and seeds. Proportion of these has a high variability depending on fruits maturity, grape cultivars, as well as the technological processes applied. Studies conducted to obtain its elemental profile revealed carbon as the most abundant (54%), followed by oxygen (38%), hydrogen (6%), nitrogen (2%) and traces of sulfur (0.08%) [11].

According to Eurostat [12] updates, the central European country of Romania, with a total area under vines of 183,717 hectares, ranks fifth among the EU member states in this economic domain, and the annual production was of approx. 974 thousand tons of grapes in 2019 according to FAO database [13]. General characteristics of Romanian vineyards and widespread cultivated varieties, together with particular pedoclimatic conditions will be presented in the next sections. Native Romanian varieties of *Vitis vinifera L.* (*i.e.* Feteasca Neagra, etc) will be presented in detail, together with their valuable properties.

Transition from conventional to organic agriculture is one of the main goals of the European Union, the aim is to continuously improve the quality of the environment and life. Organic agriculture, by eliminating the systemic treatments with pesticides and fertilizers, has the potential to generate agricultural products with low risk of contamination, safer for human and animal consumption, and implicitly may lead to revitalization of biodiversity worldwide [3, 5, 7]. Currently, the vine is one of the most widespread crops and is grown mainly in various temperate regions around the world and a minority in some tropical areas. On the other hand, *Vitis vinifera L.*, an extremely valuable crop, represents a significant source of income for many countries worldwide, and an expansion/adaptation of this crop even in the northern countries of Europe, where the climate is not friendly, is expected in the next years. Also, pedoclimatic conditions are related with technological and phenolic maturity as a result of a grapes adaptation to the environment.

Valorization of by-products generally requires a specific evaluation of composition and biological activities. Also, the recovery of valuable compounds from grape-based waste is an emerging issue in the context of circular economy, and should be performed in the most eco-friendly manner. Suitable extraction techniques and cost-effective analytical laboratory procedures need to be developed and applied.

The use or re-use of vines parts for so-called nutraceuticals, or cosmeceuticals, or other consumer-goods applications, are basically centered on phytochemical and microbiological characterization. The diversity of collected data (phytochemical, spectroscopic, others) are used in chemometric strategies for predicting a qualitative response for many applications. In the context described in the above, the information and experimental results presented in this chapter aim at providing useful data and tools, as it was graphically suggested in **Figure 1**.

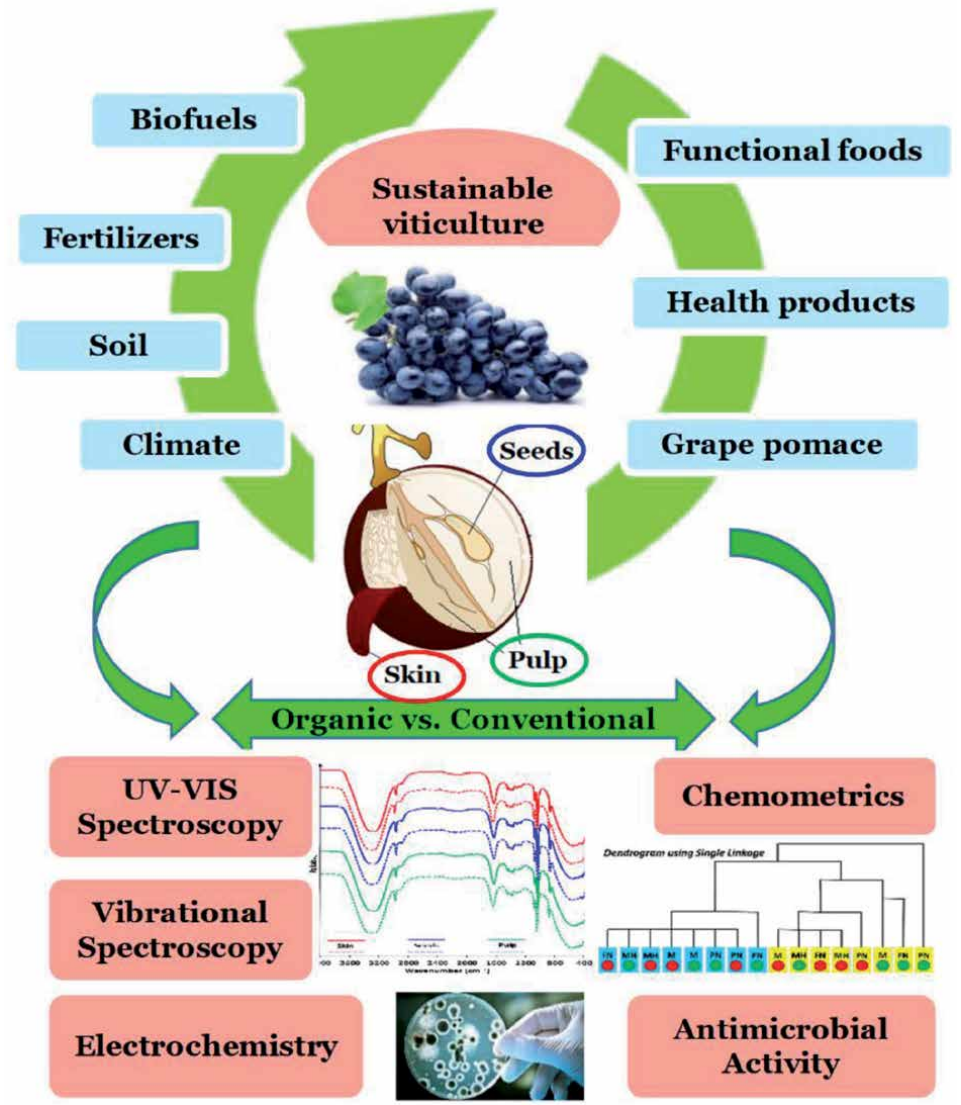


Figure 1.
Graphic representation of the research concept on Vitis vinifera L. varieties.

2. Organic and conventional vineyards in Romania: general characteristics

Vitis vinifera varieties are the most cultivated worldwide due to their high quality of fruit for wine production. However, its high susceptibility to many pests, fungal diseases and extreme temperatures is a major problem in the cultivation of vines around the world. It is of a significant importance that cultivated varieties are well adapted to abiotic and biotic stressors with different characteristics, such as cold resistance, short-term growing season, and pest's resistance. A current challenge in oenology is to obtain varieties that are resistant to grapevine diseases, without losing the quality of the grapes. In this sense, a new ecological approach to viticulture is desired, which should emphasize the organic production of grapes, recognizing the importance of the interactions of the vine (*Vitis vinifera*) with the microbial

communities of the soil. Due to different treatments in the field of viticulture [14], distinct microbial communities can form, and they may affect the potentially beneficial interactions of the soil, as a habitat, with the vines. Therefore, the scientists are currently concerned and working on identifying differences in community structures of landscape fungal and bacterial soil communities and to relate them to the type and duration of soil management and vineyard habitats [15, 16].

In the last years, the organic cultivation of *Vitis vinifera* has grown steadily in many areas, and thus in the European Union (EU) at the end of 2011, there were over 200,000 hectares cultivated in this system, corresponding to about 15% of the total crops [17, 18].

From the organic culture point of view, *Vitis vinifera* is a part of a complex agroecosystem where many organisms coexist and interact, and systemic treatments are completely missing [19]. Organic viticulture recognizes the importance of interactions between soil and plant microbial communities [16, 20], as they influence the growth, physiology, and yield of the vine.

In conventional culture, negative effects may appear on plants and soils due to application of fungicides [14], soil acidification due to fertilizers use [21, 22], and tillage [20]. The pesticides significantly affect soil microbial communities, including beneficial species such as mycorrhizal fungi [14], thus changing the interactions between vines and microorganisms and finally, modifying the phytochemical profile of grapes.

Fungicides are the main pesticides used in conventional viticulture, while copper-based fungicides (Bordeaux mixture, copper fungicide - a mixture of 20% copper and 80% neutralized copper sulfate) are the only effective methods allowed for organic viticulture. However, prolonged use of copper can also have profound effects on microbial communities, as copper accumulates in the topsoil after fungicide application [23]. Copper becomes mobile in soil pH of 5.5–6.5 and thus more available to organisms, which can create stress for microorganisms and affect their enzymatic activities [14, 23]. Also, tillage and fertilization [22], as well as weed-type wild plant communities, which grow in vineyards, especially between vineyards [15] influences the physicochemical and microbial properties of the soil. In contrast, low-input measures of organic viticulture may provide better conditions to support a higher diversity of beneficial microorganisms in the soil (*i.e.* mycorrhizal fungi) [15, 16]. These measures can avoid the selection of taxa that tolerate high levels of nutrients [24]. This is of a significant importance for vines, as they are characterized by low root densities, and this is an indication of the need for a strong dependence on interaction with beneficial root endophytes [25]. Organic vine growers recognize the importance of vine interactions with soil microbial communities. However, the lack of knowledge on this topic may affect the production, and further research on this topic is beneficial [15]. Some studies have shown that, in organic viticulture, copper-based fungicides that replace chemical pesticides may have serious effects on bacterial diversity and community structure [15, 26]. Similarly, copper has been reported to affect fungal communities in vineyards [26]. Other studies [15] have shown that the same copper concentrations were found in the vegetative parts, especially in grapes grown in both organic and conventional culture if the latter is properly managed and there is no historical accumulation of copper in the soil. The conclusion was that cupric fungicide was not the main driving force behind the differences observed in the microbial communities that formed in the two types of vine crops [15]. A recent patent [27] describes a method where the use of synthetic products for phytosanitary treatments is prohibited, and plant health is ensured in a preventive manner, only products based on simple mineral salts (copper, sulfur, sodium silicate) or plant extracts are allowed, within the limits of the rules established by legislation (EC Regulations 834/2007, and 889/2008).

Several advantages deriving from the application of the above-mentioned invention are mentioned, including the obtaining of natural grapes without chemical residues.

Vine varieties (*Vitis vinifera L.*) have been cultivated in Romania for more than 2000 years. According to the OIV report from 2018, Romania registered an increase of 10% in vineyards since 2000 [28]. In the recent years, there has been a relatively rapid increase in areas planted with wine grape varieties, such as Cabernet Sauvignon, Merlot, Chardonnay, Riesling, and Pinot Noir, along with local varieties (Feteasca, Cotnari, Busuioaca, Incense, etc.), widely cultivated in Romania [29]. Currently, Romanian vineyards have no problems with phylloxera pests, and most of the planting material propagates through cuttings. However, in cold regions, the vine is usually grafted on cold-resistant rootstocks.

The most difficult issue in quality evaluation of both organic (complies with the rules of organic farming, and is certified by a control and certification body) and conventional vineyards is the aspect related to the pedoclimatic environment (zone, climate, and soil). A recent paper [19] revealed the importance of internal (grape genetics, rootstock) and external factors (pedoclimatic conditions), that together with cultivation techniques lead to obtaining the grapes colored in the right point, rich in sugars, high aromas, and extractive compounds. In this regard, Romania, by geographical position and climatic conditions, offers good adaptability, short and perfect acclimatization of various grapes varieties. Also, it offers particular conditions of soil for high resistance of the wine against phylloxera and other diseases. These aspects contribute to the increase of vineyards quality and productivity. It is well known that cultivation of grapes for wine production, as well those dedicated to consumption as fresh fruits, is mainly done in the hills with slopes, with different altitudes, and particularly, with an open valley, ventilated by winds [30–33]. Plains and mountains are also suitable places for vine growing. Most of the vineyards in Romania are positioned on the gentle hill slopes (*e.g.,* between 5% and 25%), this being the best solution in terms of temperature, isolation, and brightness. Also, this favors the chlorophyll photosynthesis in leaves and allows the formation of sugars. On the other hand, the continental climate, with thermal amplitudes, characterized by long and hot summers and cold winters, favors a good ripeness of the grapes. However, an issue still remains - the daily thermal, because allows the accumulation of bioactive substance in the grape skins, and thus conferring a complex and elegant aroma, and fixed acids in the pulps. The average annual temperature is 11.3°C and the annual rainfall is approx. 642 mm. The distribution of rains during the vegetation period is uneven, reaching a maximum of precipitation between May and June. Summer is long, autumn is mild and dry, thus the ripening process of grape and the accumulation of bioactive compounds in varieties is the highest. The climatic parameters fully evaluated by the enoclimatic aptitude index have very good values, corresponding to a very good oenological potential. In certain harvest years, the vineyard has exceptional enoclimatic aptitude, and this happens with a frequency of 1 to 7 years. Hail is a phenomenon that may cause significant damage. In addition, the soil texture, and its composition, including pH, influence the quality of vineyards. The soil in Romania mainly consists of clay ground (absorbs water and gradually transfer it to the roots), silt (has characteristics of both clay and sand), and sand (confers porosity to the soil), and with a various granulometry. In this respect, Romanian soils that are suitable for vineyards are classified [34] in the following main categories: (1) *calcareous-clay soils* with calcareous subsoil (suitable for grapes/wines with a highest quality, with intense and varied aroma, rich in mineral notes, finesse, and longevity) characteristic for the hills towards to mountain area; (2) *clay ground* (suitable for red grapes/wines, very intense color, richness, softness) characteristic to hills to plain area; (3) *sandy ground* (suitable for grapes/wines light/

pale and transparent color, with smooth tannins, fragrant) characteristic for the plain areas towards Danube Delta. One may conclude that the Romania's fifth rank in the EU in terms of vineyards surface is strongly related to the great variability of the hydro-physical properties and soil trophicity existing in the country. These facts determine different degrees of favorability for the vine cultures, and thus obtaining of very differentiated productions in quantitative and qualitative aspects, according to the vinifera combinations/cultivated rootstocks.

Romania has an important abundance of *Vitis* germplasm resources, widely distributed throughout the country [19]. These native, old varieties cultivated in Romania (*e.g.,* Feteasca Neagra, Feteasca Alba, Tamaioasa Romaneasca, Grasa de Cotnari, Galbena de Odobești, Busuioaca de Bohotin - Tamaioasa hunata de Bohotin, Busuioaca Neagra, Riesling de Banat) or table varieties such as Victoria, Argensis, etc., have strong resistance to vine diseases, good climatic adaptation, high resistance to humidity and low resistance to light [35]. Native species are characterized by a thick dark red market, which leads to the production of ruby red wines, traditional, appreciated, with special aromas. On the other hand, Romanian native vine varieties have a significant range of volatile compounds compared to varietal flavor (polyphenolic and flavonoid compounds), a high concentration of anthocyanins, a low tannin content and considerable acidity, a rich content of vitamins and sugars, and thus may be an attractive option to produce single-variety wines [19].

3. Grapes as functional foods

Foods that promote human health and well-being are core segments of fast-moving consumer goods, with a growing awareness of the food-health relationship among consumers around the world. Due to the richness and variety of bioactive substances contained in grapes and their positive effects on human health, they are an important raw material for various applications.

Grapes from varieties cultivated in Romania contain significant concentrations of phenolic compounds with a strong antioxidant activity [36]. The *Argensis* variety offers the properties of low sugar content and high acidity, and is very appreciated in the diet of diabetics [37].

Some other studies of recent years [38–44] have aimed at studying bioactive compounds that are present in food, and have properties that may contribute to protection against chronic diseases.

A significant interest for the potential health effects of some phytochemicals such as flavonoids and other polyphenolic compounds was noticed in the last period. Thus, potential health benefits of compounds such as isoflavones and/or resveratrol etc. have been evaluated against cardiovascular diseases [45, 46], cancer [47–49], osteoporosis [50], and cognitive decline [51]. The potential mechanisms and food safety issues have been discussed in relation to their potential health contribution.

The presence of phenolic compounds in the diet has been a negative feature for a long time, if they reduce the availability of nutrients, leading to a low nutritional value of food. Since the 'French paradox' was identified, and highlighting that a moderate consumption of red wine (rich in polyphenols) contributes to lowering the rate of cardiovascular morbidity among the French population, special attention was paid to the study of phenolic compounds as food ingredients [52]. Currently, numerous studies indicate that the presence of phenolic compounds in food is important in terms of their antioxidant stability and antimicrobial protection [52–54].

Innovation in the field of functional foods must constantly guarantee the safety of products [55]; contributes to the improvement of the nutrition - health relationship, by substantiating it on a scientific basis; contributes to the conservation of biodiversity and the sustainable development of the food sector [56–58].

The sanogenic effects of polyphenols depend on the amount consumed and their bioavailability [59, 60]. The bioavailability of polyphenols is the subject of various research, in particular on intestinal absorption and influencing factors (chemical structure – e.g., glycosylation, esterification and polymerization, food matrix, etc.). According to the World Health Organization report published in 2003, over 50% of the population of Europe, North America and other industrialized regions have used complementary natural medicines at least once [61]. Regarding to the sanogenic effect of polyphenols in grapes, even if there is a series of research in this field, there is still a wide range of untapped information [62, 63]. On the other hand, taking into account the multitude of foods, with synthetic chemical compounds that become toxic to the body, especially when certain substances reach the systemic circulation, it is desired to find new natural and non-invasive solutions such as "health-protective foods", beneficial for various diseases often caused by pollution, an accelerated pace of life, uncontrolled eating [64, 65].

Starting from the practical uses of grapes, as food, their bioactive compounds and derived products are associated with the prevention of many pathophysiological processes, including cardiovascular and neurodegenerative diseases, tumor diseases, diabetes, and other illnesses. A correct and complete understanding of phytochemical compositions and antimicrobial activities of different anatomical parts of grapes from *Vitis vinifera L.,* as well as differences resulted from the variety and/or the culture management system, may lead to developing new applications, much more specific, from a wide spectrum already known. Thus, recent studies [19, 40, 66–69] have shown a direct relationship between the therapeutic benefit, chemo-preventive effects (anticancer) and the red grapes consumption, in various forms. The role of the bioactive compounds (*e.g.,* proanthocyanidins, anthocyanins and other flavonoids, hydroxycinnamates, and stilbenes such as resveratrol) has been investigated, and antioxidant, antimicrobial, antitumor effects have been found, as well as anti-inflammatory properties, and inhibiting lipid peroxidation. Thus, the use of expression 'health-protective biomolecules' in relation with these compounds looks appropriate.

A lot of attention was paid in the last period of time, both in research and development in the food industry, to functional foods and beverages, formulated with natural ingredients, with certain and scientific substantiated target physiological functions. Some of the functional beverages existing on the market include grapes and their derived products as source of biological active compounds. Not in the last, dairy products and meat products are ideal matrices [62].

Grape products, such as grape juice and grape skin extract, can be incorporated into yogurt, resulting in an increase in the content of phenolic compounds and antioxidant capacity. The degree of acceptability by consumers, from sensorial point of view, was high, aspect important in terms of product marketability [56, 58, 62].

Phenolic compounds are widely distributed in grapes [30, 54, 63]. The phenolic composition of a single grape variety depends on the anatomical part (whole grape pulp, skin or seeds). Grape extractable phenolic compounds represent 10% or less in pulp, 60–70% in seeds and 28–35% in skin. The phenolic content of the seeds can range between 5% and 8% by weight. Grape seed extracts are very good source of proanthocyanidins (usually oligomers and polymers of polyhydroxy-flavan-3-oils, *i.e.* catechin and epicatechin), many in the form of gallate or glycosides [30, 70].

About 75% of the world's grape production is destined for the wine industry, so that grape pomace is an abundant by-product of the wine industry. In total, residual

skin, seeds and stalks forming pomace represent approximately 25% of the total weight of the grapes used in the winemaking process [50]. In fact, grape pomace consists of two fractions: pomace without seeds (residual pulp, skin and stalks) and seeds [50]. Both fractions are rich in bioactive compounds, such as phenolic compounds [37].

The most abundant phenolic compound in pomace is represented by anthocyanins concentrated in the skin, respectively flavonols present especially in seeds, ranging from 56 to 65% of the total. Recent studies have shown the potential for recovery of phenols and antioxidant fibers from skin, respectively of seed oil from pomace [64, 71]. Considering that phenolic compounds are the most important secondary metabolites with antioxidant properties in grapes, the total content of phenolic compounds in grape pomace extracts is usually well correlated with their antioxidant activity [30]. Extracts obtained from pomace can be used in food, pharmaceuticals, cosmetics and other products in the form of liquid extracts, concentrates or powders [64]. Grape pomace extracts have been used as food protection factors due to their antioxidant capacity, prevention of lipid oxidation in fish products, and antimicrobial activity against various bacterial strains, such as *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter coli*, *Escherichia coli* O157: H7, *Salmonella infantis*, *Listeria monocytogenes* ATCC 7644. Bactericidal effects against mesophilic aerobic bacteria, lactic acid bacteria and enterobacteriaceae was showed by the seedless grape pomace products [33].

A high antioxidant capacity of the grape pomace flour sustains the delayed lipid oxidation, this property being by high interest in the context of concerns regarding the use of natural antioxidants in foods, in order to find out an alternative to the widely used synthetic ones.

Grape pomace extracts have nowadays a wide range of applications, from fortified beverages and yoghurts and use as ingredient in osmotic solution to obtain dehydrated fruits with high phenolic compounds to cosmetic applications. Not in the last, the extracts obtained from grape pomace were successfully incorporated into edible chitosan films, both hydrophobic and hydrophilic, providing antioxidant properties and prolonging life of the food products [44, 58, 64, 65, 71]. Grape seed extracts, rich in polyphenols, have been used to reduce the formation of acrylamide during the Maillard reaction [53].

Cosmetics with grape polyphenols are currently marketed, such as day or night cream and face serum from Pure Super Grape® (Marks and Spencer - UK), mattifying, anti-wrinkle and anti-wrinkle protection fluid from Caudalíe® (France). There are few brands in the field of food supplements that claim to use polyphenols, mainly resveratrol, from grapes. For example: 100 Natural®, Nature's Way®, Maximum Strength®, GrapeSeedRich®. These products confirm the commercial potential of bioactive compounds extracted from grapes or grape by-products [65, 72]. Some studies showed the differences in phenolic compounds concentrations in grapes anatomical parts. Thus, phenolic compounds concentration in seeds (70%) is higher than in skin (20%) and in pulp (10%) [73].

Recent research has evaluated the use of pomace flour from grapes and seeds, respectively, in various products such as popcorn, cereal bars, biscuits and cookies, extruded snacks and muffins, resulting in high-fiber products with antioxidant potential and consumer acceptability.

Pinot Noir grape fiber can be used as an alternative source of antioxidants and dietary fiber when added to yogurt and salad dressing, not only to increase the content of fiber and phenols, but also to delay the oxidation of lipids during storage, expanding shelf life of these products.

The addition of grape pomace fiber to unconventional products, such as cod and seafood, has led to a minimization of changes in flavor, color, texture and oxidation

of lipids during freezing. The antioxidant dietary fiber in grapes added to chicken breast burgers and fish muscles has led to improved oxidative stability and free radical scavenging activity [62, 73].

According to some authors, a percentage between 2 and 5% of the grapes weight is represented by grape seeds that constitute approximately 38–52% of the solid waste generated by the wine industry. In general, grape seeds contain about 40% fiber, 10–20% lipids, 10% protein, phenolic complexes, as well as sugars and minerals. About 80% of the sugar-free dry matter of the grape seeds consists of indigestible fractions, mainly cellulose and pectins [30, 73].

Grape seeds are highly appreciated for the nutritional properties of their oil, known as rich source of unsaturated fatty acids (oleic and linoleic), and phenolic compounds [73]. Grapes seed oil is widely marketed in some countries, and is used for years in numerous applications, especially in cosmetics formulations [41, 62, 71]. However, recently reported data have confirmed its promising bioactive properties and new specific uses for obtaining organic products.

Grape seeds contain 8–15% (w/w) oil with a high content of unsaturated fatty acids (oleic acid and linoleic acid), which represent more than 89% of the total essential fatty acids. Linoleic acid is an essential fatty acid receiving a lot of attention, together with the conjugated linoleic acid, due to their biological effects. Thus, recent studies have shown the beneficial effects of the grape seed oil, such as hepatoprotective, neuroprotective action and in reducing the level of cholesterol in the liver [42, 46–48].

In the food industry, grape seed oil can promote lower production costs, as it is more competitive compared to other types of oil in economic terms, and may be a new food source for human consumption. In addition, grape seed oil has a high burning point, which is why it can be considered as a potential biodiesel [11].

Food industry is constantly searching for new strategies that may lead to inhibition of the spoilage microorganisms growth. Recent studies focused on new natural compounds with antimicrobial activity capable to replace classical chemical preservatives. Several products obtained from grape pomace, in particular from grape seeds, have been proposed to act as food spoilage control additives.

The growth of mesophilic aerobic bacteria, lactic acid bacteria, *Pseudomonas* and psychotrophic populations in pork pate was delayed by the incorporation of grape seed extracts, which showed a higher antimicrobial action compared to other natural extracts (obtained from tea, seaweed and chestnuts).

Grape seed extracts showed bactericidal effects against *Escherichia coli* and *Salmonella typhimurium* and delayed the growth of *Listeria monocytogenes* and *Aeromonas hydrophila*. Incorporated in films, grape seed extracts showed a slight activity against *B. thermosphacta*. Grape seed extracts were also effective in cheese inoculated with *L. monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica*. The concentrations required to observe the antimicrobial effect were higher than in the *in vitro* tests, which suggested a decrease in the antimicrobial effect when the extracts were added to food. This inferior effect can be explained through a reduced solubility of extracts in certain foods and the interaction of polyphenols with other food components too. Grape seed extracts have a higher activity of inhibiting microorganisms, compared to the extracts obtained from the skin of the same grape varieties.

The antimicrobial effect of the grape pomace products is usually attributed to different phenolic compounds. Several studies have shown the predominant role of the phenolic acids (mainly gallic acid, followed by p-hydroxybenzoic and vanillic acids) compared to flavonoids. In this respect, gallic acid has been shown to be the strongest antimicrobial agent in grape seed extracts [53, 54]. Although the effect of inhibition of spoilage and pathogenic microorganisms by grape extracts has been

widely studied, there is still some research that highlights the ability of products obtained from pomace to promote activity or protect probiotic microorganisms against various external factors.

The effect of phenolic compounds on the growth of lactic acid bacteria may have a significant variation, depending on the chemical structure and concentration of each phenolic compound, the species of microorganisms, their growth in the environment and the growth phase. Some authors found that pomace and grape seed extracts have promoted the growth of *Lactobacillus acidophilus* [62].

Procyanidin extract from grape seeds has shown anti-obesity properties in animal and human studies. Recent studies suggest that procyanidin extract from grape seeds has a protective effect on intestinal permeability, but the mechanism is still unknown. The extract has been reported to have anti-inflammatory and antioxidant properties and the ability to modulate the intestinal microbiota. Based on these properties, it was supposed that the mechanism of intestinal barrier function mediated by procyanidin extract from grape seeds is associated with reducing the inflammation and changes within the intestinal microbiota [42, 74].

Some *in vivo* studies have shown that bioactive compounds from grapes skin improve the glutathione metabolism and reduce the apoptosis. The grape skin powder promoted the regeneration of glutathione and the reactivation of glutathione-dependent antioxidant enzymes, helping to maintain redox homeostasis and protect the intestinal mucosa against apoptosis in a model experiment of ulcerative colitis. All the fractions obtained from the skin of the grapes were equally useful for restoring homeostasis in the colon. It has been suggested that dietary fiber and grape-associated polyphenols are much more effective compared to extractable polyphenols to protect the intestinal mucosa from ulcerative lesions [45, 51, 74].

Recent research work using a system of ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC–MS/MS) on Tannat grape skin extracts showed that the main polyphenols constituents are flavonoids, phenolic acids and phenols. Also, the study demonstrated the bioavailability of these compounds *in vitro*, with the potential to modulate key biochemical activities involved in the pathogenesis of diabetes and the control of hyperglycaemia caused by this disease [37].

4. Evaluation of phytochemical and antimicrobial properties

As described in previous chapter, various beneficial compounds were reported to be present in grapes-as harvested and grape-based products, and having roles in balancing human metabolic processes related to oxidative stress [74].

Red grapes harvested from Romanian organic and conventional cultivated vineyards have been studied, several phytochemical characteristics such as total phenolic content, total flavonoids, antioxidant activity have been determined, together with antimicrobial activity, and also information on the chemical bonding has been collected. Grape extracts from different anatomic parts that are main components of grape pomace (skins, seeds, and pulps remains) were used in experiments. Main perspective of these studies was to identify and test some possibilities to re-use the by-products generated in economic activities related to vine cultures, and also to differentiate, whenever possible, between the two types of culture management (organic and conventional).

Processes aiming at obtaining high-value added products from wastes generated by wine industry, and also evaluating benefits of organic over conventional viticulture for human health, both need phytochemical and biological data, as well as comparisons/differentiation between varieties and/or cultures characteristics. In

the following paragraphs, information on the laboratory protocols and analytical instrumentation applied, together with the chemometric algorithms used to obtain complementary data were detailed.

4.1 Laboratory techniques and protocols

Different instrumental analytical techniques were reported by scientists as tools to identify and quantify antioxidants in water and hydroalcoholic extracts obtained from different grapes anatomic parts, and also for genetic characterization [3, 4, 19, 36, 71, 73, 75–78]. Top instrumental techniques such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), with various detection devices are used to obtain detailed information on the bioactive compounds profile and content, or on genetic information (geographical mapping etc). Spectroscopic techniques like ultraviolet–visible (UV–VIS), Fourier transform infrared (FTIR) and Raman, are widely used to establish the antioxidant activity of grapes samples, to identify and/or quantify classes of antioxidant species (*i.e.* polyphenols, flavonoids, etc.) and other bioactive compounds, as well as to provide raw entry data for chemometric analysis. Also, rapid electrochemical tests (*i.e.* pH, conductivity) or refraction index measurements are used to evaluate either the acidity, total dissolved solids, or total dissolved sugars in grape based samples.

Antimicrobial activity is an important characteristic for any material intended to be used in applications related to health, food or others [19, 72]. In this study, disc diffusion assay and minimum inhibitory concentration methods have been used to evaluate this property of red grape extracts against some bacterial strains isolated from natural environment, some important conclusions have been drawn and were presented below.

Considering the large-scale application of developed laboratory protocols, grapes samples were mainly characterized through spectroscopic methods such as absorption techniques of UV–VIS and FTIR, and Raman scattering. These techniques are routinely used in laboratories, and generally accepted as providing cost-effective, rapid measurements, with a convenient sample treatment, or non-destructive. Even if the recorded spectra are often not readily useable, and need data processing and analysis, further use of chemometrics may help to extract meaningful conclusions from multivariate data.

Analytical protocols included the classic steps of sampling, sample preparation, and qualitative and/or quantitative analysis. For the sampling step, grapes samples of four varieties were harvested from Romanian vineyards (out of which one was a native wine variety) as described in previous published works [3, 4, 19, 75], and then representative portions from each sample were taken for further treatment. The four varieties studied were Merlot, Pinot Noir, Feteasca Neagra and Muscat Hamburg. Grape skins and seeds were dried in the oven at 40°C for 48 hours and then stored at room temperature in closed vials, while the pulp fraction was frozen and maintained at - 18°C, and defrosted in the day of laboratory tests. To obtain the grape extracts, classic maceration and ultrasound assisted extraction procedures have been applied, both at room temperature, and using either deionized water (<0.05 μS/cm) or hydroalcoholic (50%, v/v) solvents, for a total extraction time of 24 hours. For maceration, magnetic stirring at 150 rpm has been applied for the first 3 hours, and for the second method, the ultrasound field of 45 kHz has been applied for the first 30 minutes. Then, for the remaining time up to 24 hours, samples rested at room temperature, in dark and non-humid atmosphere. For dry grape skins and seeds samples a 4% (dry weight/volume dw/v) ratio was used, while for the pulp samples, the grape fraction to solvent volume was of 12% (w/v). In general, extractions using 50 mL of solvent were proved sufficient for one set of

analysis. Separation of liquid and solid fractions was performed by centrifugation at 1000 rpm, for 10 minutes, and filtration (Whatman 4).

4.1.1 UV–VIS spectroscopy

This technique uses the interaction of the light with wavelengths in the range 200–800 nm with the molecules existing in the material of interest. An absorption phenomenon appears, with non-bonding and π-bonding electrons provide the strongest absorbances. Aromatic molecules, antioxidants such as phenolic molecules, flavonoids in particular are examples of molecules where UV–VIS spectroscopy may be successfully applied. The method is considered to have a limitation in sensitivity, because of the inability to differentiate between molecules absorbing in the same wavelengths range. Samples are either scanned as they are, or prepared according to specific protocols indicating qualitative or quantitative determinations.

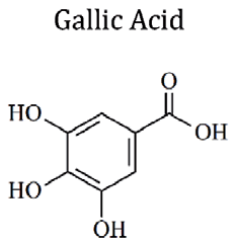
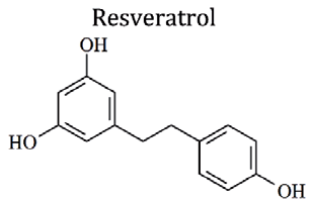
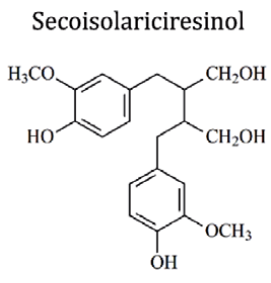
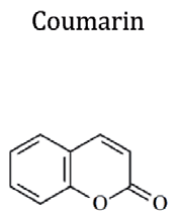
Antioxidant activity (AA), total polyphenols content (TPC) and total flavonoids content (TFC) have been determined in this study, by using UV–VIS spectroscopy.

Table 1 shows examples of antioxidant compounds that may be present in grape-based samples [4, 36, 75, 77]. As may be observed, the general structure of polyphenols contains at least one aromatic ring, with at least one hydroxyl group bonded on it. These compounds are classified considering the number of rings and the functional groups bound in the structure, and thus there are: phenolic acids, flavonoids, stilbenes, and lignans, coumarins, tannins. The health benefits of bioactive phenolic compounds have been demonstrated, and their contribution to the wine quality in terms of sensory perception (color, taste, mouthfeel, flavor, astringency, bitterness) have been recently discussed in detail [79].

With respect to flavonoids structure, in **Figure 2** one may observe that it contains two benzene rings (A and B) and an oxygen containing pyran ring (C). Flavonoids' classification in six subclasses is generally accepted, and the difference between them is given by the oxidation level of the C ring of the basic 4-oxoflavonoid (2-phenyl-benzo-γ-pyrone) nucleus, and thus there are: flavanols, flavones, isoflavones, flavanones, anthocyanidins and flavonols. **Table 1** shows the example of quercetin which belong to flavonols sub-class. The antioxidant activity of flavonoids, as for polyphenolics in general, is due to the presence and position of the multiple hydroxyl groups in their structure.

In the following paragraphs, the analytical protocols applied to generate quantitative phytochemical data of studied grape samples will be provided.

Total polyphenols content (TPC) was determined through Folin Ciocalteu method [80], the procedure was slightly adapted for grapes samples as prepared in the present study [3, 4, 19, 75]. Folin Ciocalteu reagent consists of a mixture prepared by dissolving sodium tungstate ($Na_2WO_4{\cdot}2H_2O$) and sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$) in water, and adding hydrochloric acid and phosphoric acid. Commercial already prepared reagent may be also procured. The chemical process, occurring at basic pH, is based on molybdenum reduction from +6 (yellow) to +4 (blue) after the oxidation of polyphenols in samples. The light absorption of a monochromatic radiation of 765 nm was measured with an UV–VIS spectrophotometer. Colored liquid samples were placed in glass cuvettes with 10 mm light-path, readings were done vs. a blank sample prepared with all reagents as samples, but with extraction solvent instead of grapes extract. The calibration curve has been plotted before each measurement set of samples, with gallic acid as reference antioxidant in the concentration range of 0.01–0.08 mg/mL. Similar experimental procedures were applied for both aqueous and hydro-alcoholic extracts, different samples dilutions were used so that the linear domain of Beer–Lambert–Bouguer

Polyphenols sub-class	Sub-class representative compound (name and chemical fomula)	Polyphenols sub-class	Sub-class representative compound (name and chemical fomula)
Phenolic Acids	Gallic Acid 	Stilbenes	Resveratrol 
Lignins	Secoisolariciresinol 	Coumarins	Coumarin 

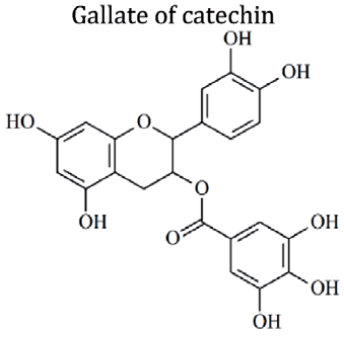
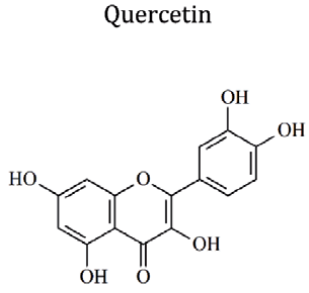
Polyphenols sub-class	Sub-class representative compound (name and chemical fomula)	Polyphenols sub-class	Sub-class representative compound (name and chemical fomula)
Tannins	Gallate of catechin 	Flavonoids	Quercetin 

Table 1.
Examples of polyphenolic compounds from grapes (names and chemical formulas).

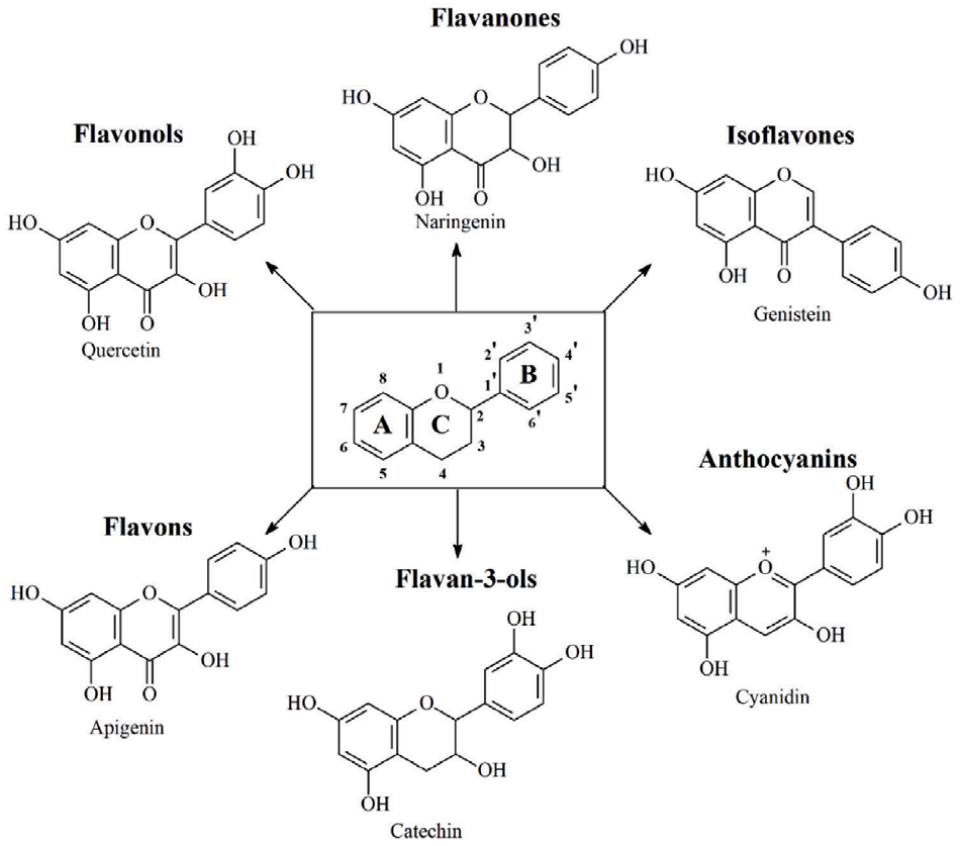


Figure 2.
General structure of flavonoids and their subclasses.

law and calibration range were reached. Final results were provided as total polyphenols content (TPC) expressed as milligrams of gallic acid equivalents per mL of grapes extract, and then reported to dry weight (mg GAE/g d.w.). All experiments were performed in triplicates and the means ± standard deviations (SD) were reported [3, 4, 19, 40, 75].

Total flavonoid content (TFC) in grapes fractions extracts was determined through the aluminum chloride colorimetric assay described in previous papers [19, 75]. In this method, some complex combinations form as products of the reaction between the aluminum ions and the carbonyl group from C-4 carbon, and hydroxyl groups from C-3 or C-5 carbons from flavonoids structure. In addition, other chemical bonding may appear between the aluminum ions and the ortho-dihydroxyl groups from A- and B- nucleus of flavonoids. All these chemical processes lead to a yellow color of the working solution, and thus the spectrometric measurement was performed at a wavelength of 510 nm, in glass cuvettes. Deionized water was used for the instrument baseline, and a calibration curve has been plotted in the range of 0.1–1 mg/mL using quercetin as reference flavonoid. Total flavonoids contents were provided as mg quercetin equivalents per mL grape fraction (skin, etc) extract. Calculations to convert the total flavonoids content in the solid grapes samples may be performed for each studied grape fraction, when needed. Analytical data were collected on triplicate samples, mean values together with standard deviations were reported [3, 4, 19, 40, 75].

Antioxidant activity (AA) of grapes extracts was evaluated by using the method involving formation of a phosphomolybdenum complex compound,

and optical densities were measured at 700 nm, in glass cuvettes with 10 mm optical path [81]. The choice of Prieto procedure was a consequence of some unsatisfactory results obtained for skin extracts when applying the 2,2-diphenyl-1-picrylhydrazyl DPPH• assay, one of the most frequently used method. It was considered that color interferences are the reason this unsuitability; as known, the DPPH• assay involves monitoring the decrease in color intensity of a purple reagent, while the tested samples (*i.e.* red grapes skin extracts) had colors in the same spectral range.

4.1.2 Vibrational spectroscopy

Two vibrational spectroscopic techniques were used during experiments, the infrared (IR) light absorption and Raman scattering, both aiming at investigating the chemical functional groups of organic compounds in studied grape samples, and potential changes occurring while applying extraction procedures. Gathering information on differences between grapes sampled from organic and conventional vineyards was also in the scope of this study.

The Fourier Transform infrared (FTIR) spectrometer used was Vertex 80v (Bruker) equipped with diamond attenuated total reflection (ATR) crystal accessory, and samples were placed on the measurement position without any additional preparation. The absorption frequencies were recorded in the mid infrared range of 4000–400 cm^{-1}, the average spectrum of 32 scans (with baseline and atmospheric correction), was declared an experimental result, and considered for further data processing. Same IR scanning procedure was followed for each of the studied samples.

Raman spectra for studied samples have been recorded with a Xantus 2 (Rigaku) spectrometer, using a light source of 1064 nm, at a power of 490 mW. The average of 5 scans (with baseline correction) was taken as the experimental result for each sample, and presented as intensity vs. Raman shift in the wavenumber range of 2000–200 cm^{-1}.

4.1.3 Antimicrobial activity determination

To evaluate antimicrobial activity of the grapes extracts, observation and quantification of the growth of several strains of bacteria isolated from natural environments during their contact with studied samples. Both disc diffusion and minimum inhibitory concentration assays were applied [3]. First, several bacterial strains were isolated from different habitats, grown in agar meat broth, and incubated at 37 ± 0.2°C, then characterized by classical microbiological techniques. These bacterial cultures were used to prepare inocula for the antimicrobial testing, colonies from 24 h-old plates were picked, suspended in appropriate media, and aerobically grown at 37°C for 24 h. It worth mentioning at this point that all the operations related to antimicrobial activity determination were performed according to a lab-protocol that avoided contamination (*i.e.* manipulations under UV light, etc).

For the disc diffusion method, a volume of 20–50 µL of fresh bacterial culture with the optical density at 600 nm between 0.2 and 0.4 was spread on Petri dishes with the media. Sterile 6 mm paper disks were impregnated in the grape extracts for 1 h, then placed on the Petri dish at approx. 15 mm from edge, and at 30 mm distance between each other, and in the end incubated at 37 ± 0.2°C for 2 days. One considers a sample as having antimicrobial activity, if after the above-mentioned incubation time, a clear area (halo) may be observed on the inoculated Petri dish around the disk impregnated with the respective sample.

The minimum inhibitory concentration of grape extracts was determined as the lowest concentration of the sample that completely inhibited the growth of tested microorganisms, as visually detected by the normal human eye. The incubation time considered was 48 h at 37 ± 0.2°C, and control samples without grape extract were tested in each set of experiments.

4.1.4 Chemometric methods

In is well known that chemometrics is generally applied to provide additional information to the direct interpretation of experimental data collected through various laboratory techniques. The usefulness of chemometrics may arise from both its descriptive approach *(i.e.* finding relationships and structure of the systems), and from the predictive one (modeling of some chemical properties, so that new properties or specific behavior may be predicted).

Several chemometric methods have been applied during the study, as valuable tools aiming at a further interpretation of the instrumental analytical data. In this respect, we may list herein the multiple linear regression, bivariate correlations of data (on the basis of Pearson coefficients), and the SPSS classification through hierarchical cluster analysis. Also, multivariate analysis and corresponding methodologies have been applied to process large data sets generated by the vibrational spectroscopic used for samples characterization [82]. Other techniques like principal component analysis (PCA), agglomerative hierarchical clustering (AHC) and discriminant analysis (DA) were also applied in this study [38, 39, 83–85], as well as combinations between them [70, 82, 86].

The Statistical Package for the Social Science v24.0 software for MS Windows (SAGE IBM® SPSS®) was used when measured phytochemical parameters and antimicrobial activity were taken into consideration for data analysis. The significance of differences between various experimental groups was evaluated at 5% level of significance.

For statistical analysis of spectral data, the XLSTAT software, 2021.1.1 version has been used (©Addinsoft, USA). First, Box-Cox transformation [82, 87, 88] was applied to obtain approximately normally distributed values. Then, principal component analysis (PCA) was used to reduce the dimensionality of the spectral data to a smaller number of components. The analysis of the score plots (FTIR and Raman data) for the first three principal components (PCs) was based on the partial bootstrap method [89], in order to estimate the proximity between the observations and to know which observations are significantly different from each other. Agglomerative Hierarchical Clustering (AHC) was performed using the Euclidean distance as the distance measure and single linkage (Ward's method) strategy to link clusters within the data set [76]. Discriminant Analysis (DA) was applied considering that when the number of variables exceeds the number of samples, one method of multivariate discrimination is to use principal components analysis and then to perform canonical variates analysis [83, 84]. Combining both PCA and DA approaches, in so called PC-DA model, leads to improving the efficiency of classification, as this procedure automatically finds the most diagnostically significant features [85, 86, 90].

Beyond the technical details of their specific application on the data recorded by laboratory and instrumental techniques, these chemometric methods aimed to complete the direct interpretation of the analytical results. Thus, additional information regarding potential correlations between the potential valuable compounds that may be extracted from studied grape samples, their antimicrobial activity, and the vineyard management type, grape varieties, or grapes anatomic parts used to prepare the studied extracts, etc. was of a significant interest once one started to apply the chemometrics.

4.2 Analytical data and results interpretation

By using the lab-investigations protocols, together with data processing and analysis using the chemometrics as described in previous sections, important information on the grape-based products from Romanian vineyards, either of organic and/or conventional type. Synthetic data were presented in this sub-section, together with cross-references where details of the research may be found. However, at the moment of submission of this chapter, some experimental data are the subject of articles being drafted or under the review process, and may be consulted in the near future.

Phytochemical characterization of extracts prepared from grapes parts harvested from Romanian vineyards (organic and conventional management types) confirmed the variability described by the literature [91–93]. As examples, the type of vineyard management, the extraction solvent and/or method influenced the TPC, TFC, AA, pH, or conductivity of some prepared extracts, while for some others differences were not significant [3, 4, 19, 75].

Table 2 presents some phytochemical parameters of grape skin, seeds and pulp (hydroalcoholic extracts obtained by room temperature maceration) of Feteasca Neagra variety of *Vitis vinifera L.,* harvested from both organic and conventional vineyards. One may observe that, for this grape variety, the total phenolic content, total flavonoids content and antioxidant activity in the extracts prepared from dry seeds is higher than in dry skin, and than in pulp. Also, once the vineyard type is considered, significant differences between the two types of culture management (organic/conventional) were recorded for TPC, TFC values of skins, seeds, and pulps, while for the AA, seeds extracts only showed significant differences. Some statistics are also provided in this table, with regards to grape varieties (a), and to phytochemical characteristics of extracts (b).

For the Pinot Noir variety, in the aqueous extracts prepared from organic grape skins a total flavonoids content of 0.317 ± 0.035 mg Quercetin/mL, almost triple than same extracts prepared from grapes originating from a conventional vineyard (0.109 ± 0.034 mg/mL), when the extraction method was classical maceration. For the case of ultrasound-assisted extraction, the TFC in organic grape skins aqueous extracts was over two-fold higher than the same kind of extracts but prepared from conventional cultivated grapes (recorded values were 0.297 ± 0.028 mg Quercetin/mL, and respectively 0.139 ± 0.074 mg Quercetin/mL) [4]. The use of hydroalcoholic solvent showed similar behavior, in the sense that TFC was higher for samples from organic vineyards, than from conventional vineyard, but to a lower extent [3].

Phytochemical parameter [unit]	Vineyard Type	Grape parts studied		
		Skin	Seeds	Pulp
TPC [mg GAE/g]	Organic	71.98 ± 4.04[ab]	150.92 ± 4.87[b]	0.88 ± 0.06
	Conventional	22.17 ± 0.58[ab]	64.48 ± 1.36[b]	0.39 ± 0.02
TFC [mg Quercetin/g]	Organic	87.72 ± 5.95	158.36 ± 11.10	8.29 ± 0.04
	Conventional	47.02 ± 2.87	122.14 ± 7.18	8.29 ± 0.05
AA [mg Ascorbic Acid/g]	Organic	23.99 ± 2.16[a]	286.58 ± 10.47	14.81 ± 0.04
	Conventional	23.82 ± 2.62[a]	157.07 ± 9.31	11.12 ± 0.02

[a] *Significant difference ($p \leq 0.05$) among grapes' varieties.*
[b] *Significant difference ($p \leq 0.05$) between vineyard type, with regards to phytochemical characteristics of extracts (one-way ANOVA, Tukey test).*

Table 2.
Phytochemical characteristics of Feteasca Neagra variety grapes parts (hydroalcoholic extracts).

For two studied grape varieties a different behavior was found when the extraction procedure in water as solvent was applied. Thus, for Merlot (wine variety) and Muscat Hamburg (table grapes) aqueous extract, regardless the extraction method at room temperature (maceration and ultrasound-assisted), significant differences were recorded in the pH and conductivity measurements, when the vineyard type was considered. For the Merlot variety, pH and conductivity of the organic grapes skin extracts were always higher than for the conventional vineyard harvested samples, while for Muscat Hamburg variety an opposite variation was found for the pH (lower for organic originating samples extracts that for conventional ones), while no notable differences were found for conductivity values [4]. The explanation of these findings could be inferred from correlations with the specific treatments used in the vineyards, according to the management type of each culture [3, 4, 19, 75, 94], and further research is desirable.

For all studied grape varieties, regardless the solvent used in the initial step, the extracts prepared from dried seeds had higher values of TPC, TFC and AA than extracts prepared from dry skins and from grape pulp, regardless the vineyard type where the samples originated, and regardless the extraction method, if either maceration or ultrasound assisted, at room temperature [3, 4, 19, 40, 75].

For the hydroalcoholic extracts, while for the grape skins extracts TPC, TFC and AA had close values with regards to the vineyard type, if either organic or conventional, for the grape seeds' extracts, the experimental findings show significant differences between the organic and conventional samples, for these three phytochemical parameters, for the wine-type grapes (Feteasca Neagra, Merlot, Pinot Noir), while for the table grapes variety (Muscat Hamburg), the values were similar. The ANOVA algorithm, and the technique of multiple comparison applied on these measured values confirmed the differences between the antioxidants content ($p < 0.05$), and stated that TPC is the parameter the most influenced by the vineyard type, for both skins and seeds of studied grape varieties [3, 40].

A series of experiments were conducted aiming at evaluating whether the extraction procedures applied lead to obtaining samples with compounds that may have antimicrobial properties. Control samples without grapes extracts were tested for each set in the same conditions with the studied grapes extracts. Several bacteria strains were first isolated from ordinary environments, characterized and stored according to standardized procedures, and then used during the tests [3, 19, 40]. It was found that hydroalcoholic extracts prepared from grape skins originating from conventional type of cultures had a significant antibacterial activity against strains of *Lactococcus*, *Bacillus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Micrococcus*, when compare to extracts obtained from the same varieties, but from grape skins originating from organic type of vine cultures. Another important experimental finding was that, when the hydroalcoholic solvent was used, the extracts of grape seeds from organic vineyards showed a broader spectrum of antibacterial activity than the seeds extracts from conventional vineyards grapes. Highest values of the antimicrobial activities, in seeds hydroalcoholic extracts, were found for the organic varieties of Merlot and Muscat Hamburg, and for the conventional Pinot Noir variety [3, 19, 40]. Antimicrobial activity data were subjected to statistical analysis, aiming at identifying correlations with phytochemical quantitative data [3, 40].

The mid-infrared spectroscopy with Fourier transformation (FTIR) has been used to obtain spectra of studied samples, in the wavenumbers range of 4000 cm^{-1} to 400 cm^{-1}. **Figure 3** shows some examples of the spectra obtained for the native Romanian variety Feteasca Neagra, on hydroalcoholic extracts prepared from three anatomic parts of grapes harvested from organic, and respectively from conventional vineyards. As may be observed in this plot, measurements results are spectra with important similarities. Thus, all FTIR spectra showed strong peaks at 3275 cm^{-1},

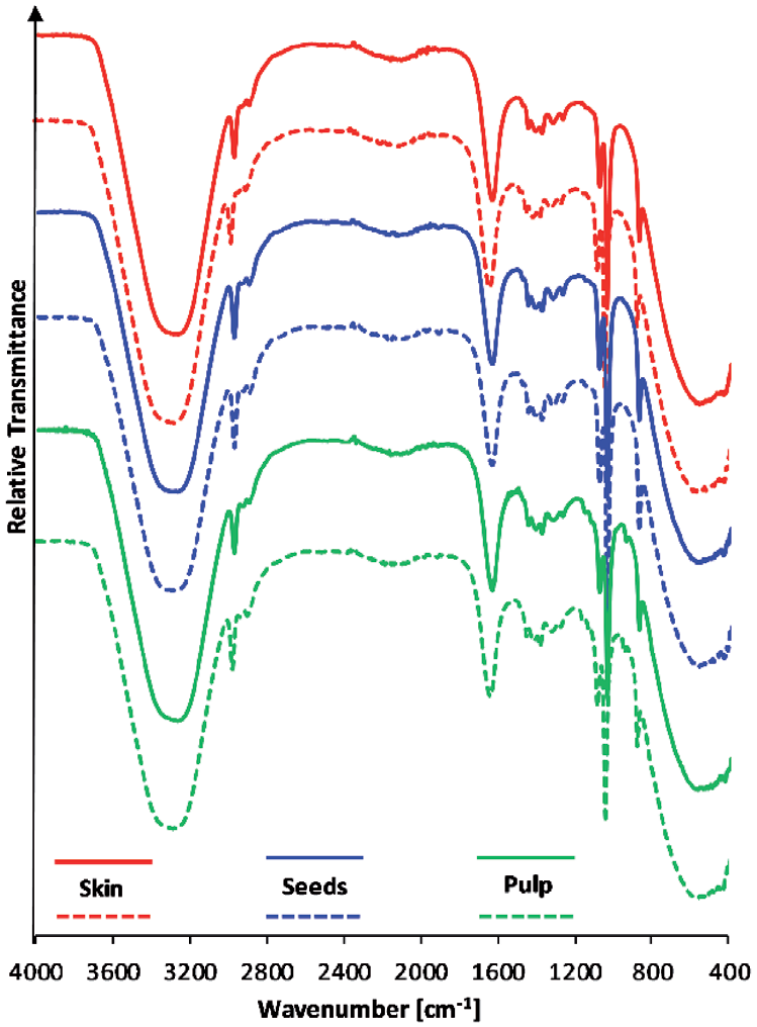


Figure 3.
Mid-infrared (FTIR) spectra recorded for grapes anatomic parts from organic (solid lines) and conventional (dashed lines) cultures of Feteasca Neagra vineyards (hydroalcoholic extracts).

assigned to O-H stretching vibration, and in the range 1043–1055 cm^{-1}, that may be assigned to C-O stretching, and to stretching vibrations of O-H and C-OH. Also, the peaks of 2979 cm^{-1} and around 2900 cm^{-1} could be assigned to asymmetric and symmetric stretching vibrations of -CH-, $-CH_2-$. $-CH_3$ from carbohydrates. The signal in the range of 1635–1643 cm^{-1} can be assigned to the aromatic C=C stretching vibrations which may correlate with the presence of anthocyanins, and also to C=O stretching vibration, while this finding may correlate with the presence of flavonoids like flavonols, flavons, isoflavones or flavanones. The peak recorded at 877 cm^{-1} was associated with the aromatic cycle C-H bending vibrations [4, 75, 77, 94]. Similar behavior was recorded for extracts of other grape varieties, provided by both organic and conventional vineyards, and are the subject of paper under review.

Unfortunately, information on some production parameters such as the irrigation level, crop yield, others, were not available for this study. Thus, further research will be considered, aiming at evaluating to what extent the recorded phytochemical data relate to the organic/conventional cultivation system only, and/or to some specific agronomic practices.

As may be observed in **Figure 4**, similar spectra were obtained by using Raman spectroscopy, and the additional data processing and data analysis through chemometric techniques have been useful to extract further conclusions, and will be detailed below.

However, given the limited conclusions that may be extracted from the direct interpretation of the infrared and Raman spectra recorded for studied samples, chemometric methods have been applied considering the spectral data. Some results were published [94] and the following paragraphs will present some statistical analysis of samples indicated in **Table 3**, together with the additional information they could provide for the experimental findings. Codes indicated in this table correspond to those indicated in **Figure 4**. Multivariate analysis has been applied to FTIR and Raman spectral data recorded for hydroalcoholic extracts obtained from the four red grapes varieties indicated in the table, and for the three grapes parts studied - skin, seeds. and pulp.

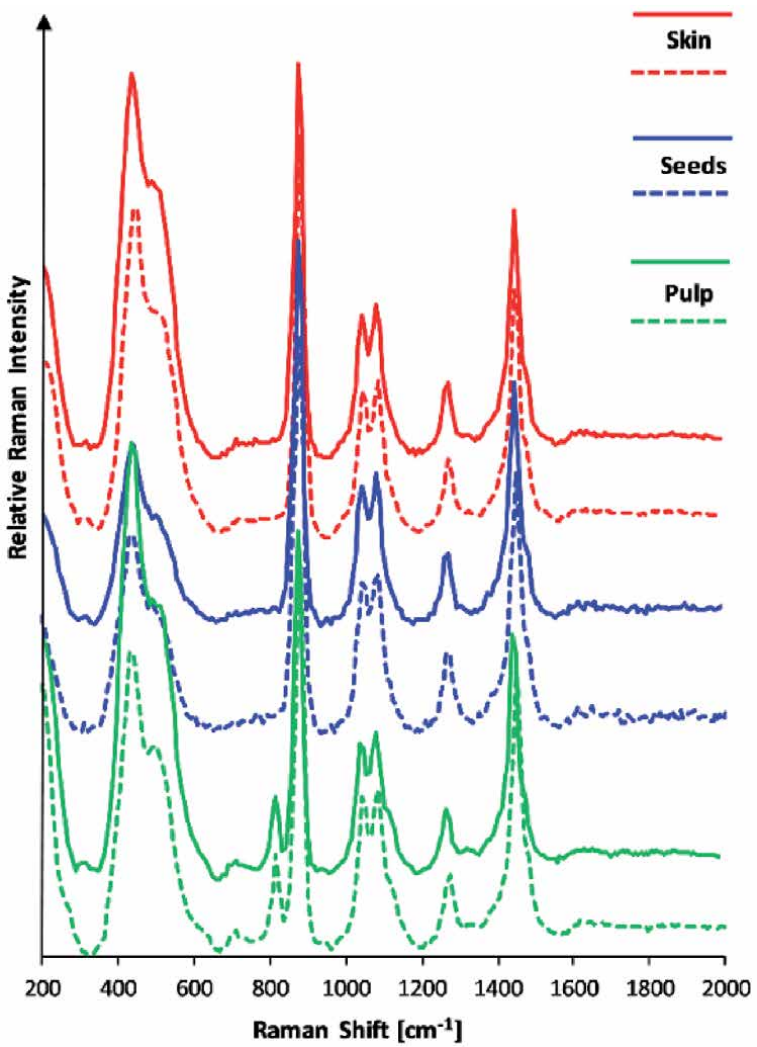


Figure 4.
Raman spectra recorded for grapes anatomic parts from organic (solid lines) and conventional (dashed lines) cultures of Feteasca Neagra vineyards (hydroalcoholic extracts).

Grape variety	Vineyard type	Sample code
Merlot	Organic	M-O
	Conventional	M-C
Feteasca Neagra	Organic	FN-O
	Conventional	FN-C
Pinot Noir	Organic	PN-O
	Conventional	PN-C
Muscat Hamburg	Organic	MH-O
	Conventional	MH-C

Table 3.
Samples codes used in the chemometric analysis of spectral data.

For the easiness of reading, conclusions extracted from statistical analysis were presented graphically in **Figure 5**. As may be observed, the figure shows information on the classification based on vineyard type, and the color and shape codes are explained in its caption. The work flow of the statistical analysis was as described in previous section.

A notable finding was that the decomposition of both FTIR and Raman spectral data through PCA revealed that with the first three principal components (PCs) a percentage higher than 90% of the total variability (the sum of percentage of variability explained by that PC and the preceding one) of the analyzed data was included. The PCA score plots showed that the investigated red grape varieties (*i.e.* skin extracts) overlapped (bootstrap ellipses) at different extent in all plots, and thus incomplete separations between varieties were noticed. However, it can be distinguished a separation between vineyard types (organic vs. conventional) for same grape variety (i.e., M-O vs. M-C, FN-O vs. FN-C, PN-O vs. PN-C and MH-O

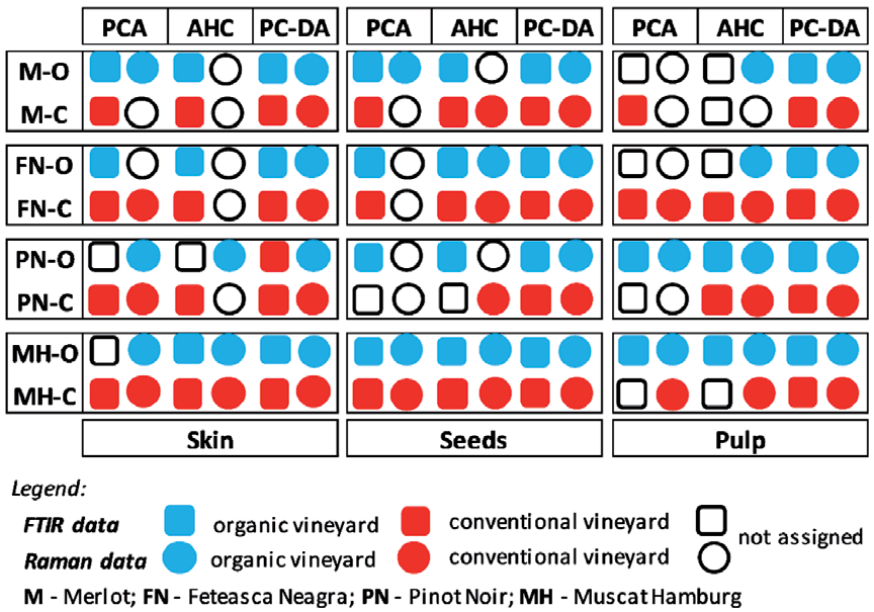


Figure 5.
Statistical classification of the red grapes hydroalcoholic extracts (skin/seeds/pulp), based on vineyard type (organic/conventional).

vs. MH-C). The interpretation of the PCs loadings, for both FTIR and Raman spectral data, revealed the spectral regions/peaks that allow the differentiation between organic and conventional vineyards for same grape variety. Similar findings were recorded for the red grapes seeds and pulp extract studied.

Further analysis performed using Agglomerative Hierarchical Clustering (AHC) allowed a clear view of the similarities and differences between red grape parts extracts. For instance, AHC derived from grapes skins, FTIR data has grouped both organic and conventional extracts into two main classes/clusters (variance decomposition for the optimal classification: within-class 97.2%, between-classes 2.8%); at a lower dissimilarity level subclusters division allow a classification based on vineyard type (excepting PN-O), a differentiation was found for each grape variety between organic and conventional vineyards. From the classification obtained by using AHC based on Raman spectral data, organic and conventional extracts were similarly included into two main clusters (variance decomposition for the optimal classification: within-class 77.7%, between-classes 22.3%). Subclusters division based on Raman data shows notable differences between organic and conventional vineyards excepting Pinot Noir variety. The AHC algorithm applied on both FTIR and Raman data for seeds and pulp extracts lead also to grouping in two clusters, for both organic and conventional vineyards.

In the end, after the application of PCA on FTIR and Raman datasets, the first three principal components scores were retained for further analysis – classification and cross-validation through PC-DA. The result was, for all the three grape parts studied (skin, seeds, pulp) that all the extracts have been correctly classified through PC-DA, with only one exception (PN-O/FTIR data for skins).

For the case of the native Romanian variety Feteasca Neagra, and considering the vineyard management type only as criterion (conventional/organic), one may observe in **Figure 4** that application of AHC algorithm on FTIR data may provide a classification for all grape parts extracts (except FN-O/pulp) of the while the FTIR spectral data allow classification through, while application of the same algorithm on Raman data, a classification is possible only for seeds and pulp extracts. Another conclusion that may be extracted from **Figure 4**, is that once the PC-DA method is applied, a classification may be obtained while using both infrared and Raman spectroscopy datasets.

5. Conclusions

Romania is one of the major vine growers in the European Union, and in the same time, concerned with expanding the application of the principles of the circular economy in this field, with positive economic, social and environmental impacts on long term. The pedoclimatic conditions in the country offer the possibility of obtaining vine productions of an important variability, with qualitative and quantitative benefits. Subsequently, the composition of grape-based direct products (wine, food and beverages, others) and by-products (grape pomace, others) may vary, and thus leading to the desirable market variety. Extracting high-added value components from wastes in the vine-related industries may be a significant action in this context. Also, application of organic type of management to vineyards has the potential to significantly contribute to the sustainability in this field.

This chapter presents useful tools on how to characterize grape-based products extracts, and offers information on some cost-effective techniques suitable to collect, process and interpret experimental data. Thus, the information provided may contribute to taking informed decisions with regards to valorization of by-products generated in vine cultures.

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Author details

Cristina Mihaela Nicolescu[1*], Marius Bumbac[1,2], Cristiana Radulescu[1,2], Lavinia Claudia Buruleanu[3], Radu Lucian Olteanu[1], Laura Monica Gorghiu[2], Gabriela Teodorescu[3] and Carmen Georgeta Holban[4]

1 Valahia University of Targoviste, Institute of Multidisciplinary Research for Science and Technology, Targoviste, Romania

2 Valahia University of Targoviste, Faculty of Sciences and Arts, Targoviste, Romania

3 Valahia University of Targoviste, Faculty of Environmental Engineering and Food Science, Targoviste, Romania

4 University of Agronomic Sciences and Veterinary Medicine of Bucharest, Doctoral School, Bucharest, Romania

*Address all correspondence to: cristina.nicolescu@valahia.ro; cristina.nicolescu.profa@gmail.com

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Chapter 7

Table Grapes: There Is More to Vitiviniculture than Wine...

Ana Cristina Agulheiro-Santos, Marta Laranjo and Sara Ricardo-Rodrigues

Abstract

Table grapes are fruits intended for fresh human consumption due to their sensory attributes and nutritional value. The objective of this chapter is to review the existing knowledge about table grapes, including a description of different varieties, with particular emphasis on the new highly appreciated seedless varieties. Following an introductory note on the world distribution and production of table grapes, also considering the impact of climate change, selected varieties of table grapes will be characterized in terms of their physiology, postharvest features, and consumer preferences. A morphological description of each variety, with emphasis on grape skin, grape rachis and grape cluster will be included. A final note on the drying of table grapes into raisins, and the most appropriate varieties for drying, will be given. The major changes occurring throughout the growth, development, and ripening phases of table grapes production will be discussed, regarding both physical (skin color and skin and pulp texture) and chemical (phenolic compounds, sugar content and acidity) parameters, as well as growth regulators.

Keywords: grapes, varieties, seedless, raisins, quality, consumer

1. Introduction

Table grapes are destined for fresh human consumption because of their sensory, nutritional, and commercial attributes, which is in line with the definition adopted for table grapes by the *International Organization of Vine and Wine* [1] "A fresh grape, produced from special vine varieties or vine varieties cultivated for this purpose and destined for consumption as such, basically because of its sensory and commercial characteristics."

The consumption of the grapes can be fresh, or derived products such as juices, wines, raisins, and has increased due to the identification of beneficial compounds for human health in its constitution [2].

The culture of the vine is one of the most important agricultural crops in the world. The world production of grapes intended for all uses, in 2018, was 77.8 million tons, 57% wine grapes, 36% table grapes and 7% dried grapes [3]. In 2018 the world production of table grapes was 27.3 million tons (**Figure 1**).

In 2020, the world area planted with vines for all purposes, wine, juices, table grapes and raisins, is estimated at 7.3 million hectares. An apparent stabilization hides the reduction in the vineyard surface in Iran, Turkey, Portugal, Uzbekistan, and USA. The leading countries in 2020, were Spain, France, China, and Italy, respectively with 13.1%, 10.9%, 10.7%, 9.8% of vineyard surface area (**Figure 2**) [4].

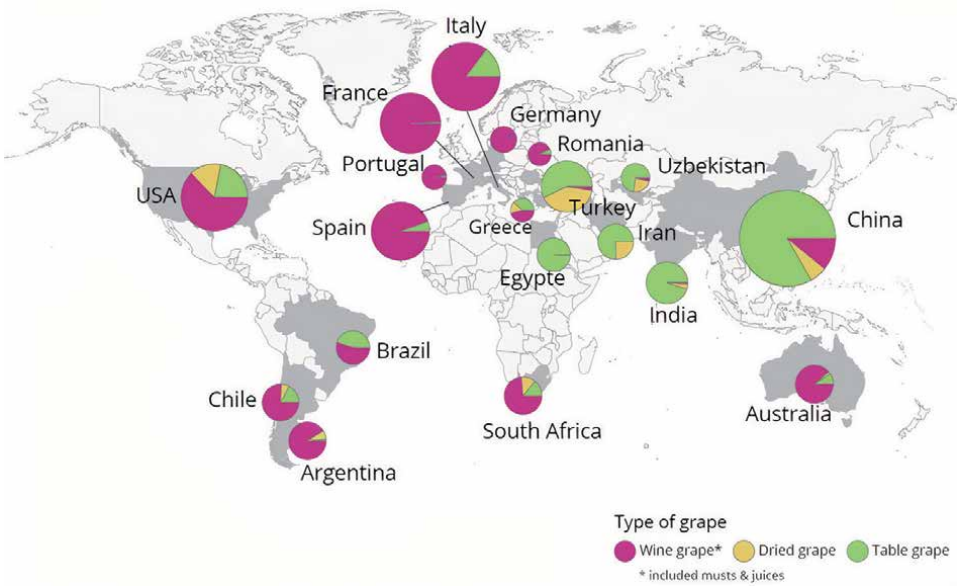

Figure 1.
Major producer countries by type of grape in 2018 [3].

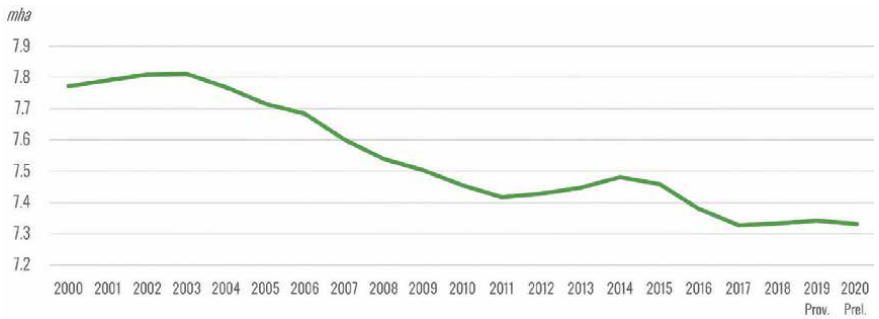

Figure 2.
Evolution of the world vineyard surface area from 2000 to 2020 (in million hectares) [4].

The world consumption of table grapes has increased in recent decades, and there has been an increase in consumer demand for high quality table grapes [5]. According to the *International Organization of Vine and Wine* (OIV) in the beginning of this century, a continuous growth of areas under table grape production was observed until 2017. Between 2007 and 2009 there was a notable growth trend in the production of grapes for fresh consumption, of about 10% (**Figure 3**) [5].

A more detailed analysis of the world table grape production, considering the 2018/2014 ratio, shows an overall value of 2% and allows us to verify that Latin American countries like Peru and Mexico show an important production increase (respectively 0.8% and 0.5%) and Uzbekistan, USA, Brazil, South Africa, Greece, Spain, and Australia show more modest increments between 0.1 and 0.3%. In contrast, Turkey and India show even slight decreases in production [4]. In 2019, Europe produced 1.7 million tons of table grapes for fresh consumption, and ten years ago the production was more than 2.0 million tons (https://www.cbi.eu/market-information/fresh-fruit-vegetables/table-grapes/market-potential).

In the year 2018, China was outstanding as the world's leading table grape producer, producing 9.5 million tons of grapes. The second ones were Turkey and India producing 1.9 million tons each, as it can be appreciated a much lower value.

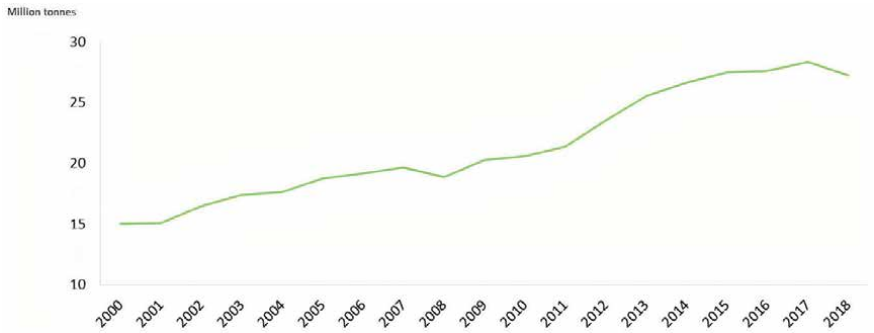

Figure 3.
World table grapes production from 2000 to 2018 in million tons [3].

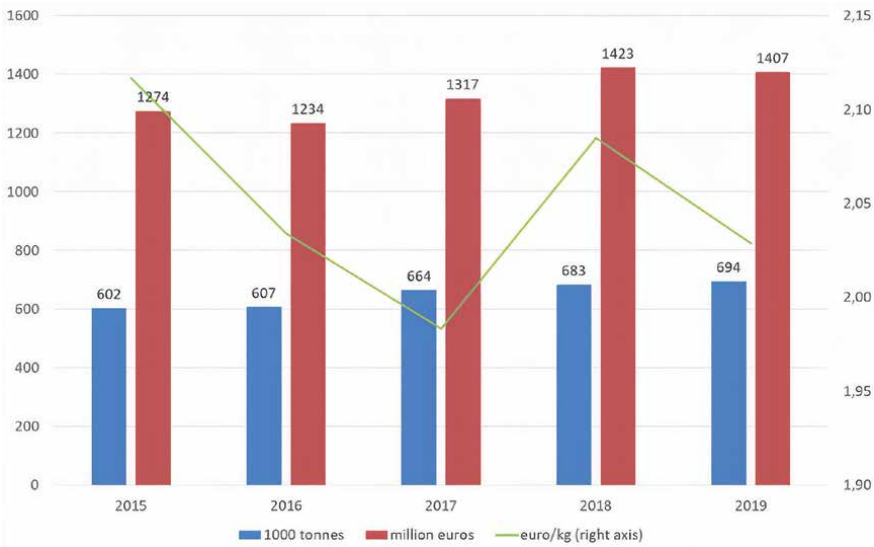

Figure 4.
European imports of table grapes from non-European suppliers (https://www.cbi.eu/market-information/fresh-fruit-vegetables/table-grapes/market-potential).

Another important observation is the increasing exportation of fresh grapes from Chile, Peru, and Turkey, developing countries and new producers. Chilean season starts in December until April, and the USA, China, Netherlands, UK, Korea are main markets for Chilean grapes due to the cycle opposite to that of the Northern Hemisphere.

The import volume of table grapes from non-European suppliers has gradually increased from 602,000 to 694,000 tonnes between 2015 and 2019, corresponding to a value of 1.4 billion Euros in 2019 (**Figure 4**).

The main grape producing countries worldwide are Italy, France, USA, Spain, and China. The more non-European significant grape exporters in the international market were Chile, and USA and in Europe, Italy. On the other side, in 2018, only three countries in Europe imported 25% of the grapes traded globally, namely United Kingdom, Germany, and Netherlands.

The existence of market strategies can be perceived as the introduction of new varieties to different products, a goal to be reached by producers, mainly important producers and companies that are gaining prominence in the international market. Big grape companies are anticipating consumer preferences of seedless grapes and sustainable packaging, for large markets, such as the United Kingdom and Germany.

The crisis caused by the Covid-19 pandemics, turned 2020 into an abnormal year, regarding international trade, and dramatically decreased the demand for table grapes in the supermarkets, for example in Germany and in the UK. This crisis serves to test the resilience of the sector and to be expecting new opportunities in the value chain [6].

The *International Organization of Vine and Wine* is committed to achieve excellence towards environmental sustainability objectives on socio-economic and socio-cultural aspects, in support of the United Nations (UN) 2030 Sustainable Development Goals (SDGs) [4]. In their Strategic Plan 2020–2024, the *International Organization of Vine and Wine*, among the various proposed axes, highlighted the first three:

Axis I - Promote environmentally friendly viticulture, facing climate change through mitigation and adaptation activities.

Axis II - Promote economic activity in accordance with the principles of sustainable development and growth and globalization of markets.

Axis III - Contribute to social development through vitiviniculture.

In the 2020/2021 season, the global production of table grapes is estimated to be maintained at 25.7 million tons, although production in key productive regions, such as Chile, Europe, and the United States substantially decreased, mainly due to the increased production in China [7].

2. Grapevine: family, genus, species, and varieties

Grapevine is a hardy perennial plant, which belongs to the family *Vitaceae*. The plant is a climber with an herbaceous or twining stem, sometimes with tuberous stems, characterized by tendrils and inflorescences opposite the leaves [8, 9]. It bears fruit in clusters, and the fruit, grapes, is botanically called a berry that results from the development of the ovary of the flower [10].

The genus *Vitis* is the most representative of this family and the most interesting for the vine industry. It has over 80 identified species and is composed of two subgenera, *Muscadinia* (2n = 40 chromosomes) and *Euvitis* (2n = 38 chromosomes) [8, 10, 11]. The subgenus *Muscadinia* consists of three species, including *M. rotundifolia*, and is known by resistance to cryptogamic diseases.

The subgenus *Euvitis*, which is divided into three groups: (a) East Asia group consists of about 55 species and shows minor interest in present viticulture; (b) American group consisting of more than 20 species, including *V. labrusca*, *V. riparia* and *V. rupestris*, and shows high interest in use as a rootstock due to resistance to phylloxera; (c) Eurasian group composed of the species *Vitis vinifera* L., the most representative and planted worldwide. It consists of two sub-species: *sylvestris*, which corresponds to the wild form of the vine, and *vinifera*, the cultivated form [8, 11]. According to Creasy and Creasy [8], many of the non-*V. vinifera* species have been vitally important to the commercial development of *V. vinifera* cultivars, in finding a solution to the problem of phylloxera and other soil-related pests and conditions.

Nowadays, the genus *Vitis* presents a large genetic diversity with several thousands of varieties. However, there is a high number of synonyms (different names for the same cultivar) and homonyms (identical name for different cultivars) to be considered. Also, the number of varieties in the world is estimated at 6,000 for the *V. vinifera* species on its own [11]. Additional information on the origin, main use and pedigree of cultivars is available from the *Vitis International Variety Catalog* [12], which allows the rapid and easy comparison between molecular fingerprints.

The existing grape varieties have different features, regarding shape and size of the berry and bunch, berry tonality, organoleptic quality, productivity, among others, which give them aptitude for different uses.

Grapes can be considered for different uses according to their characteristics: (a) wine production and fermented grape products using varieties with higher acidity and moderate sugar content, (b) table grapes for fresh consumption, using varieties with low acidity, low in sugar and that meet specific standards of size, color and shape and (c) raisins, suitable varieties being seedless, with low acidity and rich in sugars [13].

According to recent data presented in the *Vitis* International Variety Catalog-VIVC [12], 53.98% of grape cultivars are used for wine production, 30.57% for table grapes (fresh consumption), 7.42% with dual suitability (wine and fresh consumption), 7.06% for rootstocks and less than 1%, more precisely 0.98% for raisins.

In table grape cultivars, berry size, firmness, sweetness, and color are important characteristics [14]. Berry size and yield are desirable in table grape vineyards, for which full irrigation is recommended [15].

However, some trials conducted by Shahidian and colleagues [16], in a vineyard of 'Crimson', with different irrigation sub-treatments with stress periods, showed significant decrease in mean berry weight, and thus in marketable fruit and also reduction in total soluble solids and an increase titratable acidity (TA), so the consequences of this stress period were a reduction in the maturity index and a delay in the maturity. The use of sap flow ratio between well irrigated reference vines and vines under reduced irrigation can potentially contribute to water savings, in order to find the level of irrigation reduction at non-critical stages of vine growth, triggering irrigation events only at a previously defined critical threshold.

The quality parameters of table grapes differ from wine berries quality parameters, and therefore irrigation practices to optimize berry quality can be quite different [17].

In wine vineyards, full irrigation is not recommended because it increases the size of the berries, which produces a decrease in the proportion of pulp in the skin, which does not benefit the quality of the wine.

Not be forgotten the use of rootstocks in grape plantation, that has become a common practice among grape growers around the world, mainly because rootstocks allow the culture to be conducted under unfavorable soil conditions, such as the presence of nematodes, diseases and pests, high salinity, among others [18, 19]. Around the world, most vineyards are grafted onto commercial hybrid rootstocks from *Vitis berlandieri*, *V. riparia*, or *V. rupestris,* which were developed at the beginning of the 20th century to control *Phylloxera* devastated European vineyards from American *Vitis* spp. [8]. The role of rootstocks in the maintenance of the crop and in the final product obtained has been studied given its relevance, particularly in aspects related to the symbiont's use of soil nutrients [20].

3. Description of the most important commercial varieties

In the last decades, the cultivation of seedless table grape cultivars has increased considerably, because consumers in many countries highly appreciate these new varieties, seedless, with firm and sweet berries [21]. However, those who think that these seedless table grapes are new are mistaken, because as early as the 19th century, William Thompson in California achieved the first significant crop, 50 pounds of seedless grapes. This breeding work has relied on varieties from Turkey and local rootstocks.

The production of seedless, i.e., apyrenic, table grape varieties has been of increasing interest, mainly because the demand in recent decades has grown, since this type of fruit is more convenient to consume [21, 22]. In addition, the selling price of these varieties is usually higher than that of seeded grapes. So, many of the

new table grape varieties that have recently come onto the market are apyrenic, and more appreciated and sought after by consumers.

In the case of seedless grapes, it is possible to distinguish two mechanisms of seedlessness depending on the time when development was disrupted: (a) parthenocarpy (observed in Corinth cultivars), which occurs when the ovary is able to develop without fertilization of the ovum; (b) stenospermocarpy (observed in Thompson cultivars), when pollination and fertilization trigger ovary development, but embryo/ovule abortion occurs 2 to 4 weeks after fertilization, and partially developed seeds or traces of seed are visible in the grape [23–25]. In seeded grapes, the transition from flower to fruit requires pollination and fertilization of the ovary for seed formation [23].

According to Costenaro-da-Silva et al. [26] and Varoquaux et al. [25] the parthenocarpy mechanism leads to the development of very small seedless and spherical berries that are usually considered for raisin production, while stenospermocarpy leads to the development of berries with dimensions compatible with commercial requirements for fresh consumption.

According to Picarella and Mazzucato [24] the term parthenocarpy is used in the broad sense to indicate both forms of apyrenia.

However, to obtain a bunch of grapes with a considerable number of well-developed berries, it is necessary to apply particular and complex hormonal treatments. Gibberellic acid is used to thin the bunch berries, elongate the bunch, increase berry size, and reduce seed traces. The concentration of the initial spray of gibberellic acid depends on the cultivar [25].

Seedlessness can also be induced by applying hormones to young inflorescences [24].

The shelf-life of seedless fruits is expected to be longer than seeded fruits, since seeds produce hormones that activate senescence [25].

3.1 Seeded varieties

3.1.1 'Red globe'

The bunches are pyramid-shaped, conical, with wings, semicircular. They can reach exceptionally large dimensions and weight. Berries are seeded large and spherical (9–10 g), consistent, the skin can be easily peeled, with a physical resistance worth mentioning that allow easy management and contribute to a long shelf-life. 'Red Globe' grapes are very sweet with a high soluble solids content (SSC) (19°Brix) and an SSC/TA ratio of 49, which makes them highly appreciated by consumers [27]. They also have a high resistance to rupture in compression, which makes them particularly interesting for postharvest handling and transport [27]. It is an early-budding variety with a long and late maturity period and a long shelf-life.

This variety is the second most cultivated variety for table grapes, covers 159,000 hectares worldwide, and 91% of the area under this variety is in China. 'Red Globe' yields between 8 and 30 tons per hectare [11].

3.1.2 'Cardinal'

'Cardinal' berries are large, spherical with a bright green color and a crisp flesh. They present a slight muscat flavor when fully ripe. This variety was introduced in Europe after the Second World War, and it is very important in the Mediterranean region. Preliminary results obtained with 'Cardinal' grapes grown in the south of Portugal have 17.5°Brix, an acidity of 0.43 g/100 g^{-1} fresh weight, and an SSC/TA ratio of 45 [27].

3.1.3 'Italy'

The berries are large, oval, with a crunchy texture, juicy, sweet, and present a very yellow color. The bunches are medium-sized and well filled out. Long harvest period until the end of the season.

3.1.4 'Palieri'

The bunches are long, medium full and have an average weight of 600 g. The berries are large (17–20 mm) and oval, with a medium-thick, firm skin covered with pruin, and the pulp is crunchy, resistant, juicy, and medium sweet (14°-16°Brix). Preliminary results for 'Palieri' grapes from the south of Portugal have 15°Brix, an acidity of 0.20 g/100 g^{-1} fresh weight, and an SSC/TA ratio of 40 [27]. 'Palieri' grapes are resistant to handling and transport due to high coefficients of apparent elasticity and firmness of the flesh. Moreover, berries are characterized by their high resistance to compression and a low resistance to rupture [27].

3.1.5 'Dona Maria'

'Dona Maria' is a Portuguese variety obtained in the 1950's in the *National Agricultural Station* in Oeiras, Portugal, and quickly spread throughout the country. It is a cross of 'Moscatel de Setubal' and 'Rosaky', their brunches are large or very large, cylindrical, rarely winged. The berries are very large, elliptical in shape, with a yellowish green color when ripe [28]. The skin is resistant, covered with a thick layer of pruin, and the pulp firm and succulent. It is much appreciated for its floral flavor, and very sweet and large berries. The bunch ripens in August and at the first fortnight of September coinciding with many other varieties [28]. Nevertheless, 'Dona Maria' grapes have a great commercial interest in Portugal, due to its light muscatel flavor and sweetness. Moreover, it is practically only sold in local markets. Berries are resistant to transport and keep their fresh appearance for a long period, if cold storage is done correctly, otherwise the berries of yellowish-green color turn brown [28]. The worst defect of 'Dona Maria' is the fact that the berries detach easily from the pedicels in an advanced period of maturation. If this problem is fixed, considering their exquisite flavor and long shelf-life, it could be considered a variety with potential for a wider market.

3.2 Seedless varieties

The cultivation and consumption of seedless table grape cultivars has increased considerably in recent years, by demand of the consumer who highly appreciates the absence of seeds and is willing to pay more for these sweet, firm and seedless grapes [17]. In fact, one of the objectives of the current breeding programs is to obtain varieties with good characteristics and mandatorily seedless [23]. 'Thompson Seedless' is the main source of seedlessness for breeding programs around the world, while also being an important commercial seedless variety for consumption [26].

According to FAO and OIV [29], the criteria to select the new varieties, among other, are: the presence or absence of seeds, shape, color, skin thickness, maturity period, resistance against diseases and pests, capacity to be transported without damage and shelf-life.

Although it seems impossible to list the current commercialized varieties of table grapes, a summary description of those considered to be the most significant, taking into account the following criteria: the varieties with commercial/economic

importance, worldwide; the varieties that are very innovative and those that, in our opinion, will be prominent in the future.

According to Fortes and Pais [30], the traditional varieties of *Vitis vinifera* table grapes are: 'Alphonse Lavallée', 'Bastardo Ruzo', 'Dominga', 'Moscatel Negro', 'Muscat of Alexandria', 'Ribier', 'Thompson Seedless'/'Sultanina', 'Tinta Pais'. The modern varieties are 'Autumn Royal', 'BRS Morena', 'Cardinal', 'Crimson Seedless', 'Flame Seedless', 'Guibao', 'Italia', 'Michele Palieri', 'Moscatel Italica', 'Muscadoule', 'Muscat Hamburg', 'Napoleon', 'Otilia', 'Perlon', 'Red Globe', 'Superior Seedless' [30]. Moreover, some table grape varieties resulting from traditional and modern inter-specific crossing or other species of *Vitis* have been described, namely: 'Alachua', 'BRS Clara', 'Campbell Early', 'Canadice', 'Delaware', 'Eudora', 'Flouxa', 'Honey Seedless', 'Janet', 'Kyoho', 'Nativa', 'Niagara', 'Ruby Seedless', 'Southland', 'Tamnara', 'Tano Red', 'Concord' and 'Muscat Bailey A' [30].

Recently, a group of researchers from the *Universidade de Évora-MED*, Portugal, in collaboration with a company from the nearby region, proceeded to characterize some apyrenic varieties ('Timco', 'Melody', 'Scarlota' (Sugra19), 'Alisson', 'Melissa' and 'Autumn Royal') produced under the specific climatic and agronomic conditions of a producer company located in Alentejo, South of Portugal (38°05′22.2"N 8°04′51.1"W). The obtained results are generally in agreement with those published worldwide, although with increased soluble solids content (SSC) values. Timco', 'Scarlota' and 'Alisson' table grapes had the heavier berries. 'Melody', 'Melissa' and 'Autumn Royal' were lighter ($p < 0.05$). Although 'Melissa' had the lightest berries, their caliber was higher than most. 'Alisson' has the smallest caliber berries. 'Melissa' had the lower skin firmness of all the varieties studied. There are no statistically significant differences in SCC and their values were all very high. 'Autumn Royal' had the lower acidity of all varieties studied while 'Scarlota' had the highest ($p < 0.05$). Total phenolic content was higher in 'Autumn Royal' and lower on 'Timco' ($p < 0.05$). 'Autumn Royal' showed the higher capacity of scavenging free radicals ($p < 0.05$). Considering all the results presented and the current interests of consumers, 'Autumn Royal' can be pointed out as a very interesting variety from the organoleptic and nutritional point of view. To perform shelf-life tests with this variety produced in this edaphoclimatic conditions is necessary to define an adequate marketing strategy [31].

3.2.1 'Sultanina' or 'Thompson seedless'

'Sultanina' is the first apyrenic or seedless variety cultivated in the world. The synonyms for 'Sultanina' are numerous: 'Kishmish' in Afghanistan, 'Thompson Seedless', 'Sultana', or 'White Sultana', 'Kišmiš', among others [11]. It is an ancient grape variety, originating from Afghanistan. This variety has a multiple purpose use, for drying to produce raisins, for vinification to produce wine especially in Turkey and the USA, distilled to make a spirit beverage (Raki, a typical Turkish beverage obtained by distilling fresh or dried grapes, flavored with aniseed and with an alcohol content of 45%) and also for fresh consumption [11, 32]. 'Sultanina' grapes are highly valued by customers as table grapes due to their organoleptic quality characteristics, mainly sweetness, sharpness, firmness, and light green brilliant color. The berries are seedless, elliptical, of small to medium size, 16–22 mm, and cylindrical in shape, yellowish green. Their flavor is said to be sharp, sweet (17°-19°Brix), juicy and the pulp crisp and consistent. The bunches are large, cylindrical, or conical and compact, with a very variable weight depending on cultivation practices, between 350 g and 700 g. Their small caliber can be improved trough applications of gibberellic acid. Maturation is somewhat late.

According to OIV [11], this variety is the leading variety of table and raisin grapes in the world, with about 273,000 hectares, however, the OIV estimates a decrease of vineyards. It is particularly cultivated in Middle Eastern countries and Central Asia. As extreme production values we can refer to 80 tons/hectare in South Africa [11]. This is one of the most economically important fruit crops worldwide [33, 34].

3.2.2 'Kyoho'

'Kyoho' or 'Kioho' is one of the obtained varieties in Japan before the Second World War, resulting of a cross between tetraploid cultivars of *V. vinifera* ('Ishiharawase') and *V. labrusca* ('Centennial'), very common in Japan. 'Kyoho' was first produced by a breeder named Y. Ohinoue in 1945 with the aim of making a cultivar with large berries due to its tetraploid nature. So, 'Kyoho' purple berries are large (12–14 g), easily peeled skin, characterized by edible flesh, sweetness (18–20°Brix) and a strong but pleasant foxy taste[1]. They are not or not very prone to bursting, but they detach easily from the bunches when fully ripe and have a short shelf-life. 'Kyoho' is generally a seeded grape but can produce apyrenic berries, which are obtained with several applications of gibberellic acid. The 'Kyoho' yield ranges from 12 to 15 tons per hectare [11].

'Kyoho' cultivation area reached 365,000 hectares in 2015, being the most widely grown grape variety in the world. In China more than 90% of the table grape area is occupied with this variety, in Japan, is the most produced one, and 'Kyoho' grapes are very appreciate in South Korea, China and Thailand. The Asiatic consumers appreciate the big caliber and the soft pulp.

3.2.3 'Crimson'

'Crimson Seedless' is a late apyrenic table grape variety. It is one of the most produced table grape cultivars in the world. It results from five generations of hybridizations at the United States Department of Agriculture (USDA) Horticultural Field Station in Fresno (California), and this breeding program started in 1926 [35]. The last cross was between the *Vitis vinifera* cultivar 'Emperor' and the USDA selection 'C33–199', resulting in 'Crimson Seedless' (previously known as 'C102–26' from the USDA selection) [36]. This variety is widely grown in the United States of America, precisely in the state of California, and in Europe, for example in Portugal. 'Crimson' grapes are highly valued by European consumers, who greatly appreciate their firm and crunchy texture, and their taste, which they classify as excellent, for its sweetness [17].

The berries contain inside two aborted seeds that are practically undetectable by consumers. The pulp is light yellow, translucent, fleshy, and firm. Regarding epidermis, it is thick, offers medium resistance and well adhered to the pulp [35]. 'Crimson' grapes present a medium degree of acidity, and the index of ripeness, SSC/TA ratio, varying between 35 and 40 [37]. 'Crimson' presents heterogeneously colored berries and bunches, which depreciates its external evaluation. So, to avoid this obstacle the bunches must be exposed to adequate sunlight during ripening, for this it is common to thin out the shoots and remove the basal leaves that surround the bunches increasing the sun incidence on the bunches [38].

[1] The origin of the term "foxy" is unknown. "Foxiness" refers to a unique wild grape aroma, a combination of an earthy aroma and a sweet muskiness. It is very common in *Vitis labrusca* 'Concord' American grapes. Methyl anthranilate (MANT) is responsible for this aroma, which is also found in fragrant flowers, like jasmine.

Because it is a variety with the characteristics already described and late harvesting, it becomes desirable to increase the availability of these grapes in the market for a longer period, in order to sell them during a time of low supply, when there are higher prices, which could be extremely important for producers [39].

3.2.4 'Autumn royal'

'Autumn Royal' developed by the University of California in Fresno, USA, is a seedless variety that presents large berries, which confers a high commercial value to these grapes. A recent seedless grape variety with large, conical bunches (400-600 g) and elongated, 17–22 mm, dark purple-black thin skin and crunchy skin, translucent white yellow-green and firm flesh. The thin skin hides a firm texture and a crunchy flesh with a neutral flavor and medium sweetness (14°-19°Brix). Generally, these grapes are seedless, however they can develop seed beginnings not detected by consumers.

'Autumn Royal' is a late-season grape adequate to extend the season. Ripening in the middle of summer, Spanish producers harvested this variety from mid-August to mid-September [40].

This variety is susceptible to berry cracking, and this problem has been the subject of numerous studies [41, 42]. Another negative aspect is the weak attachment of the berries to the rachis, for what it should be recommended to handle the bunches very carefully during harvest and postharvest [43].

4. Grapes and ripeness

The grapes are clustered into berries, and each cluster is made up of two distinct parts: the stalk (the woody part) and the berries (the fleshy, edible part). The stalk is composed of a main axis, the rachis (longest branch) that is attached to the peduncle, and shorter branches, the pedicels, which support the berries and provide them with water and mineral salts [44, 45].

The berry, in which the edible part corresponds to the pericarp, is the complex of tissues that surround the seeds, being constituted by three layers [10, 44, 45]:

i. The exocarp (skin) is the external part of the berry, consisting of a heterogeneous and elastic membrane that distends with the development of the berry. The constituent cells of this layer have an active metabolism, presenting a regulatory function, namely of transpiration, of other tissues of the pericarp. The compounds responsible for the color, flavor and aroma are accumulate in the tissues of this layer.

ii. The mesocarp (flesh or pulp) is composed of large, thin-walled, polygonal-shaped cells, which are apparently somewhat disorganized. This layer accumulates high amounts of organic acids and sugars in the vacuoles.

iii. The endocarp is the tissue surrounding the seeds, with more organized cells, but difficult to distinguish from the mesocarp.

4.1 Fruit growth, development, and ripeness

The process of berry development and growth has been the subject of numerous studies, it seems to be consensual that it is characterized by a double sigmoid curve, divided into three distinct stages that report to periods in which specific changes occur in berry development [44, 46, 47]:

i. **Stage I - Berry formation**

This initial phase is characterized by a rapid period of berry growth, which is due to both cell division and an increase in cell volume [43, 45]. The berry, green and firm, behaves like any other green organ of the vine, i.e., it performs photosynthesis and respiration functions [48]. Chlorophyll is the predominant pigment during this phase [47]. In this period the respiration rate is high and there is accumulation of organic acids, such as malic acid and tartaric acid, but the sugar content is reduced, since sugars are consumed during cell multiplication [43]. Cell division decreases and the number of cells becomes definitive, and the final size and shape of the berries is determined [10].

ii. **Stage II - Stationary lag stage**

This period is characterized by a decrease in the rapid growth rate of the berry and the concentration of organic acids reaches its highest level [45]. The berries remain firm, but photosynthesis, respiration rate and chlorophyll concentration decrease [43]. The determination of the maturity phase is accomplished by the duration of a phase of near stability, referred to as the lag phase [10]. The transition between phases II and III is known as *Véraison*, and described as the change in berry color in red cultivars [44].

iii. **Stage III - Berry ripening**

This final stage is characterized by a decrease in the growth of the berry, due to the cessation of cell multiplication, and the increase in volume caused exclusively by the enlargement of its cells. The ripening of the berry begins, and the loss of firmness is marked [45]. The loss of chlorophyll and the increase in the level of abscisic acid, which has an influence on the accumulation of polyphenols, leads to the white cultivars acquiring a translucent yellow and the red ones a light and later dark red color [43]. The supply of water, minerals, cations and sugars is carried by the phloem, since the xylem vessels are blocked from the moment when the berry reaches 6 to 7°Brix [47]. Sugar content increases, while TA decreases [43].

4.2 Physicochemical changes

Water is one of the main constituents of grape berries, and significant amounts are required for their full growth and development [43]. At maturity, grape berries have a water content of around 75–80% of their fresh weight [49].

Throughout berry development, water losses occur mainly due to transpiration, and this intensity depends on climatic conditions and changes during berry development [49]. Most of the water required by the fruit is supplied by the xylem until *Véraison*, but after this period the xylem vessels present in the berry are blocked and water transport is carried by the phloem, the main supplier of water to the fruit [44, 49].

Sugars result from the photosynthesis process carried out in the green organs of the vine, migrating to the various parts of the plant in the form of sucrose [49]. Until the beginning of the *Véraison*, sugars are consumed in cell growth, but also by migrating to the fruit for the growth and maturation of the seeds [44]. Sugars are the basis for several compounds, such as organic acids and amino acids, synthesized and found in the fruit [43].

Sucrose, a sugar predominantly transported in the phloem, is formed by the union of a glucose and a fructose molecule. When the sucrose is in the berry it is hydrolyzed, forming again the referred hexoses (fructose and glucose), existing in the pulp [43, 44].

At harvest, the amounts of glucose and fructose are approximately identical, varying between 8 and 12% of the fresh weight of the fruits, and after maturity there is a tendency for fructose to predominate [43]. Sucrose and other sugars are present in the fruit, but in very small amounts [43].

The main organic acids present in grape berries are tartaric, malic and citric acids, with the first two representing more than 90% of the total acids in the berry [43–45]. Tartaric acid is a secondary product of sugar metabolism and its content increases during herbaceous growth due to intense cell multiplication. Regarding malic acid, it is an intermediate of sugar metabolism and during herbaceous growth the sugar produced gives rise to this acid that is stored in the vacuoles of the pulp cells [45]. Tartaric acid is biosynthesized before *Véraison*, so the amount per berry remains stable, while malic acid is biosynthesized before *Véraison*, but also during ripening, and is degraded through respiration, which consequently leads to a decrease in its amount per berry [46, 49].

During *Véraison* and the ripening period, the berry volume increases and the membrane tension of the vacuoles in the pulp cells starts to decrease, which leads to the degradation of malic acid [43, 46].

Phenolic compounds, also called polyphenols, are organic compounds that result from the secondary metabolism of plants and are biosynthesized through the shikimic acid cycle. They are defined as substances that have an aromatic ring consisting of six carbon atoms with one or more hydroxyl groups or derivatives of this basic structure [49]. The phenolic content of plant-based food depends on intrinsic factors such as genus, species and variety and extrinsic factors such as agronomic and environmental conditions, ripening process, and storage conditions. Phenolic compounds are present in the berry since its formation, resulting from the catabolism of sugars [44]. They are synthesized in the berry, with different amounts, proportion and types in the skin, pulp, and seeds, and can vary significantly among cultivars [44, 50]. Regarding the total phenolic compounds present in the berry, it is known that in the skin the total extractable phenolic compounds are between 28 and 35%, the pulp presents values below 10% and the seeds between 60 and 70% [50]. Grape is one of the major sources of phenolic compounds in the human diet, the main classes of phenolics compounds in grapes are flavan-3-ols, tannins, anthocyanins, flavonols, hydroxycinnamic acids, hydroxybenzoic acids and stilbenes [49]. These compounds are of great interest since they have high nutritional value and protective function against diseases caused by oxidative damage, such as heart disease, stroke and cancer [49]. White grapes, when compared to red grapes, have lower total phenolics contents, partially because they do not synthesize anthocyanins in significant amounts [44]. These compounds can act as antioxidants in several ways, namely by scavenging free radicals, scavenging oxygen radicals and as chelators of metal ions [50]. Moreover, they play an important role in grape quality, since they inhibit lipid oxidation and participate in the processes responsible for color, astringency and aroma, inhibit lipid oxidation and fungal proliferation [50].

Mineral elements naturally originate in the soil and their accumulation in grape berries is accomplished via the xylem, except for potassium which accumulates via phloem [51]. These elements constitute between 0.2 and 0.6% of the fresh weight of the berry [52]. During berry growth, the accumulation of large amounts of nitrogen, calcium, phosphorus, and magnesium occurs, with the main mineral being potassium [49]. The accumulation of nitrogen and potassium is carried out before and after *Véraison*, while the accumulation of calcium, phosphorus and magnesium

is preferentially carried out after *Véraison* [46]. The distribution of mineral elements between the epidermis and pulp and their accumulation in the berry varies depending on factors such as variety, climatic conditions, and water availability [46]. The accelerated berry transpiration could be associated with higher fruit mineral nutrient content [49]. Mineral elements are highly important for human nutrition because they are not synthesized by our body, which has led to an increasing interest in studies on the constitution of fruits and vegetables in this type of elements.

There are different definitions for food texture, and it can be evaluated through sensory analysis and/or instrumental methods, which are related to the evaluation of food structure and the determination of its chemical composition. Textural attributes vary during the pre- and postharvest period, being affected by ripening stage, plant nutrition, water stress, storage temperature and relative humidity [53].

The fruit texture is dependent on the biomolecules involved in the cellular structure of the cell walls being the changes mostly attributed to changes in the composition and structure of cell wall polysaccharides [54].

With the initiating changes in fruit texture, there are modifications in the chemistry of the middle lamella and primary cell wall components (pectins, celluloses, and hemicelluloses) that accelerate the loss of fruit firmness [55, 56]. Studies conducted during storage period of grapes suggest that a reduction of cell wall pectins and hemicelluloses occurs, since during fruit ripening these undergo solubilization and depolymerization, which contributes to cell wall disintegration [56, 57]. Moreover, softening has also been associated with the flow of carbohydrates and osmotically active nutrients to the fruit due to competition for the accumulated reserves and the phytohormonal-caused differential movement of solutes [38].

According to Ejsmentewicz et al. [56], homogalacturonan (HG) is proposed as one of the main components of the cell wall, involved in the texture changes of fruits.

The importance of texture evaluation is due to the knowledge of these textural changes during ripening, and storage and with the differences found among varieties, being a quality attribute valued by consumers in table grapes.

The rheological behavior of foods is related to the deformation, disintegration, and flow when a force is applied, and the response can be evaluated as a function of force, time, and deformation. According to Abbott [58], fruits have a viscoelastic behavior when subjected to a load, so the force, time and deformation (intensity, duration and speed of the load) determine their rheological behavior.

In table grapes, the instrumental determination of the consistency of the berry epidermis and the compactness of the pulp, provides relevant information about the acceptability of the product by the consumer [59]. Grape berry texture is one of the most important quality parameters affecting the consumption of this fruit [56, 58].

According to Rolle et al. [60], from the point of view of consumer texture of table grape berry includes different attributes, mainly hardness (firmness), elasticity, shape, and sensations in the mouth during chewing.

The texture analysis is a rapid, and low-cost analytical technique, that can be applied in viticulture and enology as a routine monitoring tool for the grape quality. Previous studies have indicated that the grape texture is linked to cultivar and growing location, reflecting a terroir influence on grape quality [61, 62], and instrumental texture parameters were used to investigate the effects of vineyard practices [38, 50].

The color of the grape skin or exocarp is classified as green-yellow, pink, red, red-gray, violet-dark red, blue-black and red-black [63]. This attribute can be easily assessed instrumentally in color spaces, the most commonly used being CIELab, in which the color is defined by the coordinates L^*, a^* and b^*.

5. Quality and postharvest

The quality of a product encompasses sensory attributes, nutritional value, chemical constituents, textural properties, functional properties, and defects [58]. Consumers use their five senses - sight (appearance), smell (aroma), taste, touch (texture) and hearing to evaluate the product quality, and integrate all these senses to decide on the acceptability of the product [58].

In the specific case of consumer acceptability and quality evaluation of table grapes different attributes must be considered, which are reached in the third and last stage of berry development, and includes intrinsic (visual, mechanical, chemical, etc) and extrinsic (cultivar, production methods, country of origin, price, etc) attributes [59].

Visual characteristics and physicochemical properties are involved in sensory and quality evaluation of table grapes. The color, size and shape of the berry are the primary characteristics that consumers observe, together with taste, aroma, and texture [59, 64]. Consumers favor freshly picked, moderately dense triangular bunches, with a fresh, green-colored rachis. They also prefer grapes with juicy, firm flesh and few or no seeds [45, 59].

The firmness of grape berries is a quality parameter widely associated with the characteristic of crunchiness, and indicates that they have been recently harvested [54]. Loss of firmness is associated with loss of turgidity and physiological modifications that affect berry structure [54, 56].

The harvest date of table grapes is set by the producer taking into account the following quality parameters: SSC, TA, SSC/TA ratio and color [59].

Table grapes are considered non-climacteric fruits with a relatively low rate of physiological activity that exhibits a gradual decrease in respiration during ripening [65]. The berries exhibit very low ethylene production and low respiratory intensity, while the respiratory intensity of the rachis is 15 times higher than that of the berries [45]. Therefore, the quality of table grapes tends to deteriorate rapidly during postharvest, reducing its shelf-life.

Table grapes are subject to severe postharvest losses during the storage and long-distance transport, being mainly of physiological, mechanical, and microbial infection origin [66]. During postharvest, table grapes are sensitive to rapid moisture loss, which results in rachis drying and browning, water loss, berry shatter, and fungal infections (mainly caused by *Botrytis cinerea* Pers. and *Penicilium* spp.), the most important factors limiting their quality and marketability and causing quantitative and quality losses [40, 59, 66]. This type of disorder often occurs due to improper handling during harvest, and throughout the marketing process. However, this characteristic is very distinct among varieties, in some cases being an obstacle to its commercialization over long distances, as is the case of the 'Dona Maria' Portuguese variety. Temperature and humidity control, as well as improved packaging conditions can reduce the undesirable occurrence of fungi.

It is generally agreed that the most important and destructive postharvest disease in table grapes is gray rot, caused by the fungus *Botrytis cinerea* Pers. [45, 47]. Gray rot can originate from latent infections initiated before harvest, from spores present on the bunch, and from visibly infected berries that have not been eliminated during selection operations [47]. These, which at the beginning have a white coloration, after a few days acquire a grayish coloration, which characterizes the disease (**Figure 5**) [47].

Another disease that occurs during the postharvest period is blue rot caused by fungi of the genus *Penicillium* spp., which although less important than the one mentioned above, also causes damage during the storage period of table grapes (**Figure 6**).

Figure 5.
Magnifying glass observation of Botrytis cinerea on a 'Crimson' table grape berry using an Olympus SZ61 at 350X magnification.

Figure 6.
Magnifying glass observation of Penicillium spp. on a 'Crimson' table grape berry using an Olympus SZ61 at 350X magnification.

The contact of infected fruit with healthy fruit, leads to its contamination, so that through the existence of an inoculum in a berry, it easily spreads throughout the cluster [47]. The infection can be initiated in the vineyard, in the packaging units or during the storage period.

The most commonly applied postharvest techniques are based on the optimization of temperature and control of the relative humidity of surrounding atmosphere, as well as the development of packaging, which limits the decrease in moisture content and protects against physical damage during the entire postharvest period.

Temperatures between −1 and 1°C and 90 to 95% relative humidity are established as assertive conditions for table grapes [45, 47]. Refrigeration, associated with high relative humidity, is one of the most appropriate technologies to extend the shelf-life of fruits, since low temperatures decrease biochemical reactions, microbial activity and minimize moisture loss by reducing transpiration [67].

The commercially recommended method for table grape preservation consists of rapid pre-cooling immediately after harvest followed by sulfur dioxide (SO_2) spraying, keeping the temperature and relative humidity at these values constantly throughout the storage period, which will decrease the losses associated with this period [47]. The use of sodium metabisulfite generators is another of the table grape preservation practices commonly used in the international market, in the form of papers impregnated with the active substance, or bags with the solution or powder formulation, considering the higher the temperature and relative humidity, the faster the gas is generated [47].

Modified atmosphere packaging (MAP), associated with refrigeration, has beneficial effects in preventing weight loss, reducing metabolic activity, decreasing color changes in the berry and rachis, reducing respiration rate, decreasing microbial populations with consequent reduction of fungal incidence over shelf-life [68, 69]. Moreover, several studies have referred the use of MAP in table grapes, with perforated and non-perforated plastic films, based especially on polyethylene and polypropylene [69].

The use of controlled atmospheres (CA) is another technique used to maintain quality attributes and control postharvest losses in table grapes.

In addition to the techniques presented, it is also possible to mention the use of ultraviolet radiation (UV-C) [70], hypobaric and hyperbaric treatments [71] and treatments with gaseous ozone, ozone in water or ozone injection in the cooling chambers [72–74].

Therefore, it remains necessary to develop strategic, residue-free alternatives for postharvest quality control of table grapes that are safe for health and the environment and compatible with commercial practices.

In recent years, there has been a growing interest in the use of innovative and environmentally friendly technologies, such as edible coatings or films and biodegradable films associated with the application of natural compounds, like essential oils, that will both add value to food products and extend their shelf-life [75–77].

6. Nutritional value of table grapes

The nutritional and functional interest of grapes in the human diet makes relevant the knowledge of its chemical composition, which is very complex. Although there are differences in the chemical level for different varieties, agronomic aspects, and locations. The chemical composition of grapes (European type, such as 'Thompson seedless'), red or green, raw, is presented below in a generic way, according to the USDA *FoodData Central* (**Table 1**) [78].

In general, table grapes, like other fruits, have a high-water content, close to 80%, provide carbohydrates, mainly in the form of sugars, and are low in proteins and lipids. It also noteworthy the large quantity and diversity of vitamins, essential amino acids, and minerals, with a high potassium content.

It should also be noted that grapes are rich in different polyphenols (phytochemicals which are antioxidant compounds), which contribute to physiological and biological activity for the food industry such as antioxidant and antimicrobial activities [79, 80].

Resveratrol is a phenolic compound with antioxidant activity, present in berry skin of grapes. Analyses with 'Dona Maria' grapes revealed that this variety has high concentrations of this compound [28].

In recent years, there has been a notable increase in the interest for grape by-products, such as seeds and skins, which have nutritional properties and biological potential with nutritional and pharmaceutical application, such as anticancer, anti-inflammatory, cardiovascular prevention [79, 81, 82].

Components (per 100 g)			
Water	80.54 g	Total dietary fiber	0.90 g
Energy	69.00 Kcal	Sugars	15.48 g
Protein content	0.72 g	• Sucrose	0.15 g
Fat content	0.16 g	• Glucose	7.20 g
Carbohydrates (by difference)	18.10 g	• Fructose	8.13 g
Minerals			
Calcium (Ca)	10.00 mg	Potassium (K)	191.00 mg
Iron (Fe)	0.36 mg	Sodium (Na)	2.00 mg
Magnesium (Mg)	7.00 mg	Zinc (Zn)	0.07 mg
Phosphorus (P)	20.00 mg	Copper (Cu)	0.13 mg
Vitamins			
Vitamin C (total ascorbic acid)	3.200 mg	Folate, total	2.000 µg
Thiamin	0.069 mg	Vitamin B12	0.000 µg
Riboflavin	0.070 mg	Vitamin A	3.000 µg
Niacin	0.188 mg	Vitamin E	0.190 mg
Vitamin B6	0.086 mg	Vitamin D	0.000 µg
Fatty Acids			
Saturated fatty acids (SFA)	0.054 g		
Monounsaturated Fatty Acids (MUFA)	0.007 g		
Polyunsaturated Fatty Acids (PUFA)	0.048 g		

Table 1.
Chemical composition of table grapes. Nutritional information about energy value of the grape per 100 grams of fresh weight for organic constituents; per 100 g of dry weight for minerals [78].

7. Conclusions

Table grape production worldwide has been stable for several years, mainly due to increased production in China. In fact, conventional production areas, such as Europe or the United States, have decreased their production area in recent years. Some players in the international table grape market are gaining relevance, such as South Africa and South American countries.

Traditional table grape varieties, such as 'Red Globe' or 'Cardinal', are still commercially interesting varieties, mainly due to their extended shelf-life. However, consumers prefer seedless varieties, especially for their sweetness, such as 'Crimson' or 'Thomson Seedless'/'Sultanina'. In fact, although research on some of these varieties has been done for quite some time now, they are still a challenge to producers due to their shorter shelf-life, related to reduced viability and browning of the rachis, among other problems.

Innovative preservation postharvest methodologies need to be further developed and tested under field conditions, in a joint, collaborative effort between academia and producers, to extend the shelf-life of the most valued seedless table grape varieties.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Ana Cristina Agulheiro-Santos[1,2*], Marta Laranjo[1] and Sara Ricardo-Rodrigues[1]

1 MED-Mediterranean Institute for Agriculture, Environment and Development, IIFA-Institute for Advanced Studies and Research, University of Évora, Pólo da Mitra, Évora, Portugal

2 Plant Science Department, School of Sciences and Technology, University of Évora, Pólo da Mitra, Évora, Portugal

*Address all correspondence to: acsantos@uevora.pt

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Section 2

Fermentation and Microbiology

Chapter 8

An Overview on *Saccharomyces cerevisiae* Indigenous Strains Selection Methods

Laura Pulcini, Elisa Gamalero, Antonella Costantini, Enrico Tommaso Vaudano, Christos Tsolakis and Emilia Garcia-Moruno

Abstract

From the fundamental studies of Louis Pasteur in the XIX century to the current genomic analysis, the essential role of microorganisms in winemaking industry is well recognised. In the last decades, selected *Saccharomyces cerevisiae* strains with excellent fermentative behaviour have been widely commercialised in form of active dry yeasts. Currently, the production of organic and "natural" wines represents a new economically relevant trend in the wine sector. Based on this market demand, the use of industrial yeast starter could be perceived as non-organic practice and then, rejected. However, in order to preserve wines sensory quality, healthiness, and to avoid organoleptic defects given by undesirable microorganisms, the "yeast factor" (*S. cerevisiae* or non-*Saccharomyces*) cannot be ignored. The purpose of this chapter is to describe the methods of selection of wine yeasts focusing the attention on indigenous *S. cerevisiae* strains. In fact, the use of ecotypic yeasts may represent a good compromise between the needs of microbiologically controlled fermentation and a modern vision of wine as natural expression of its "terroir", also from the microbiological point of view.

Keywords: *Saccharomyces cerevisiae*, selection methods, ecotypic strains, terroir, wine organoleptic profile

1. Introduction

Microorganisms are of primary importance in the agri-food industry. The knowledge of the microbial metabolic processes, as well as their behaviour and their technological characteristics, are required for any transformation process aiming to obtain healthy and quality foodstuffs. Wine production is also based on this assumption.

In oenology, the availability of yeasts able to drive alcoholic fermentation (AF) process and bacteria that efficiently carry out malolactic fermentation is required. In fact, in the first phase of the wine production process the yeasts, mostly belonging to the genus *Saccharomyces*, transform glucose into ethanol and carbon dioxide through the primary metabolism of sugars. Subsequently, lactic acid bacteria (LAB), usually *Oenococcus oeni* or *Lactobacillus* spp., metabolise malate into lactate, thus reducing the wine acidity [1, 2] and avoiding microbiological alteration.

In the past, fermentation of fruit juice, like those of apple and pear to produce cider, grape to obtain wine, or grains to make beer and so on for any kind of alcoholic beverages, have carried out by indigenous and naturally occurring microorganisms present in the original "must" [3–5].

The first molecular evidence in a Chinese Neolithic village, dated back to 7000 BC, shows that the food processing activity has given rise, without awareness, to the evolution of the genus *Saccharomyces* with the formation of new species, probably by interspecies hybridization or polyploidization [3]. Referring to *Saccharomyces cerevisiae*, its genetic evolution, which is due to human manufacturing, reflects the spread of grapevine cultivation and led to the origin of numerous strains [4–6].

Since the discovery of fermented beverages, their production process has undergone many evolutions, but initially the role of the microorganisms was unknown. Only in a second moment the choice of the best microorganisms to be used in a specific production, and their genetic improvement, become a conscious option. Hence, a certain degree of genetic yeast improvement was implemented in response to the requirements of wine production processes [3]. In fact, the scientific community proposed to the industry the use of starter cultures, that could be defined as a microbial (bacteria, yeast, mould) preparation containing a large number of live cells or resting forms of at least one species/strain that once added to a raw material leads to the production of a fermented food by accelerating and driving the fermentation process. The starter culture could contain unavoidable residues of additives and culture media [7–10].

Regarding wine production, until 150 years ago, also the transformation of grape must into wine took place without knowing the biological agent driving the fermentation process. In the usual cellar practices, it was carried out the inoculation of the must with a small amount of matrix from a previous successful fermentation, that in wine production was called "pied de cuve" [9]. In 1864, the role of microorganisms in fermentation was discovered by Louis Pasteur thus paving the way to the modern microbiology. Further research developments, achieved through microbiology, ecology, biochemistry and recently, molecular biology, have elucidated the metabolisms and in particular the biochemical process of alcoholic fermentation (**Figure 1**), as well as the interactions among microbial communities involved in winemaking, the phylogenetic and taxonomy. Based on this knowledge, the key role of yeasts in determining the quality of wine is now universally accepted [1, 11–13].

These scientific achievements have made it possible to supply oenological products and starter cultures appropriate for the industry. In fact, beginning from the mid-1960, the production and use of *S. cerevisiae* strains in form active dry yeasts (ADY) has expanded from California (United States) to the rest of the world [11–14]. In the major wine producing countries France, Italy, Spain, USA, Australia and Sud-Africa the use of ADY has almost fully replaced the spontaneous fermentation, especially in large-scale productions [3, 11, 13].

The importance of the adoption of yeast starter inoculation mainly consists in provide a faster beginning of AF. This is a stable and reproducible wine making procedure and, at the same time, ensures the absence of defects due to unwanted microorganism contamination [3, 9, 11]. The genetic selection of commercial ADY by the industry is based on the identification of specific technological and physiological features (**Table 1**) [3, 11, 15, 16].

The discovery of DNA, together with the development of molecular techniques further contributed to the taxonomic classification and, in a more practical context, to the identification of useful and spoilage microbes [17].

This also allowed the development of genetic improvement programs aiming at increasing genetic variability using diverse techniques (e.g. intra- or inter-specific

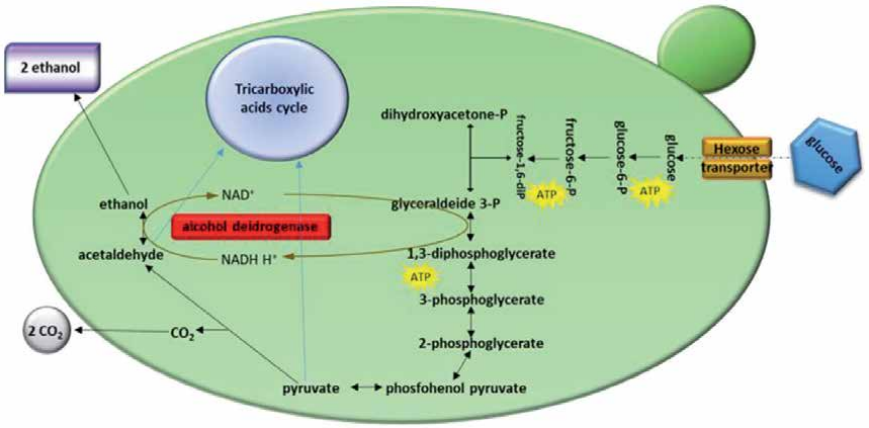

Figure 1.
Central metabolisms of alcoholic fermentation in yeasts.

Technological features	Desirable	Undesirable	Depending on process
Ethanol tolerance	x		
Complete fermentation of sugar	x		
Fermentation vigour	x		
Resistance to SO_2	x		
Type of growth in liquid media (Dispersed cells, Aggregates cells, Flocculence, Foam formation, Film formation, Sedimentation speed)			x
Growth at high and low temperature			x
Killer factor			x
Qualitative features			
Fermentation by-products (e.g Glycerol, 2-Phenyl ethyl acetate, Ethyl butanoate, Isoamyl alcohol, β-Phenylethanol)	x		
Volatile acidity, Sulphuric compounds (H_2S, SO_2)		x	
Enzymatic activity (e.g. β-Glucosidase, Esterase, Proteolytic enzymes, Carbon-sulphur lyase)			x
Ethyl carbamate precursor		x	
Effect on wine colour			x

Table 1.
General features to be considered in the selection of wine yeast.

hybridization) and by genetic engineering techniques, mainly focused on improving the yeast qualitative characteristics [18–20]. In the last decades, genetically modified yeast was also obtained by insertion of useful genetic determinants of different species in *S. cerevisiae* genome [18, 21, 22].

More recently, a new technology to engineer the genome of microorganisms, based on CRISPR/Cas9 system, has been developed. Vigentini et al. [23] applied this editing system in engineering of wine yeast to obtain genotypes with low production of urea through the deletion of DNA coding for arginine permease.

This character is important because urea represent a precursor of ethyl-carbamate (EC) which is considered probably carcinogenic to humans [23–26].

Despite these scientific developments, the current appreciation of local, natural and organic food and wines by consumers has led again to the exploitation of spontaneous fermentation [27]. In fact, organic producers and some consumers consider the use of industrial yeast starter as a non-organic or non-natural practice. Moreover, due to the use of the same commercial strain for various wine style in different winemaking geographical areas, a standardisation of wine sensory characteristics is possible and negatively considered. These criticisms are justified, but, on the other hand, a spontaneous fermentation has to deal with the risks of loss quality related to potential stuck, uncontrolled microorganism development, spoilage and off-flavour production. These problems are only partially addressed by technological strategies aimed at controlling the process [8, 9]. Another aspect to be considered is the wine safety: the uncontrolled development of unwanted microorganisms could lead to the production of toxic compounds, such as biogenic amine, ethyl carbamate or mycotoxins which could negatively impact on human health [8, 9, 28].

As reported by the International Organisation of Vine and Wine (OIV), from winemaking point of view, there is a constant requirement to improve the wine style to answer to the consumer's demand for natural products and to compete in the globalised market [29–31]. As in the past, even today the scientific answers to these new market demands can be found by moving to specific yeasts selection. Massive propagations of yeast isolated from their own vineyard in order to inoculate the must, is an alternative strategy for winegrowers that combines unique sensory attributes with safe fermentations. Furthermore, the exploitation of indigenous yeasts is emerging as a marketing plan in several wine regions because the wines are perceived with more complex taste and flavour [9, 32].

The research of wild strains of *S. cerevisiae* to be applied in wine production processes started in the late 1990s. Other studies on non-*Saccharomyces* genus are currently performed in many regions of the world [33, 34]. The research of new strains is based on the need of new genotypes coming from genetic variability. As previously mentioned, different yeast strains can develop different secondary metabolites profile, therefore providing distinct character to the wine [32, 35].

A strategy to find *Saccharomyces* spp. genetic variability is to search it in the natural biodiversity of microflora present in the vineyard. Sampling in cellars would not be very fruitful for this purpose, because cellar premises and equipment could be heavily contaminated by commercial starters [36–38].

Based on these ideas, the approach of propagation of the autochthonous yeasts for wine production encounters the consumer needs as well as the main winemakers' target: terroir-yeast in the production of more complex tasting wines with a certain stylistic distinction, while preserving quality [36–38].

The aim of this chapter is to describe the methods applied for the selection of wine yeasts particularly on the indigenous *S. cerevisiae*. The possibility of using autochthonous yeasts is an innovative approach that increases the link with the terroir and a wine stylistic distinction. Moreover, it allows to obtain greater communication and product differentiation in terms of marketing.

2. Selection program of indigenous *Saccharomyces cerevisiae* strains

Considering the oenological objectives described, the selection of indigenous yeasts must be planned and involves experiments aimed to isolate and propagate yeasts, and to test various oenological feature on laboratory and pilot scale (**Figure 2**).

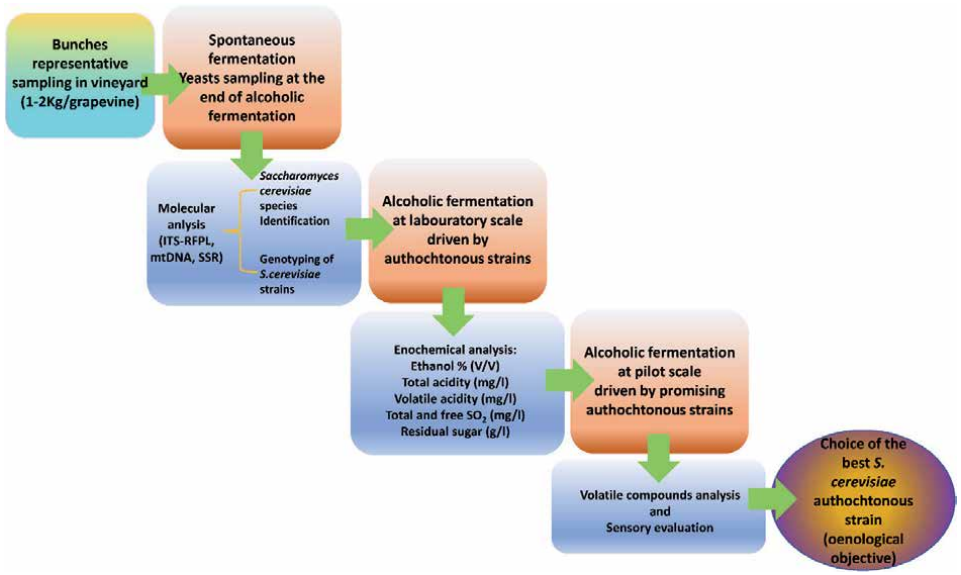

Figure 2.
Scheme of a selection process of indigenous S. cerevisiae *yeasts.*

2.1 Yeast sampling in vineyard

The vineyard soil would represent a reservoir of genetically different *Saccharomyces* spp. strains especially when the fruits are ripening and after the harvest. In fact, the increase of the number of fermentative yeasts during or near the harvest time has been recorded by molecular analysis, identification of culturable microorganisms and metagenomic approach [39, 40]. However, soil sampling at harvest time is not the optimal strategy for the isolation of wine yeast. The presence of *S. cerevisiae* in vineyard and at beginning of the fermentation process is sporadic [39–41]. In fact, yeasts belonging to the genus *Saccharomyces* spp. are not dominant on sound berries. The huge biodiversity of microflora living on bunch of grapes is related to insects and birds, that visit the ripe grapes [42]. *S. cerevisiae* strains are mainly detected during spontaneous fermentation when autochthonous grape yeasts and bacteria reduce their density due to the harsh environmental conditions represented by the high sugar content in must (realising a hypertonic living condition), and the increasing ethanol concentration in wine [32, 42]. To obtain an efficient selection of native yeasts, it is strongly recommended to start a spontaneous fermentation under controlled conditions [43, 44].

Several studies on spontaneous fermentations demonstrated the occurrence of an ecological succession with continuous shifts of the microbiota composition until the end of the process [42]. Due to the extreme condition of the must, especially high sugar concentration (250 g/l), low pH (3.5), nutrient availability and high osmotic pressure, the fermentative yeasts result to be more favoured compared to the species coming from the vineyard. *S. cerevisiae* is not dominant in this early step, but several fermentative yeasts such as *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Pichia* spp. and *Candida* spp. are detectable and carry on the alcoholic fermentation. The density of ethanol sensitive yeast species is reduced by the increase of alcohol concentration. *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *C. stellata*, *C. zemplinina*, *Lachancea thermotolerans* can resist at 6–8% of ethanol, while *S. cerevisiae* proliferate vigorously up to consuming all the sugar and can easily tolerate up to 15–16% (V/V) of alcohol. After three days from AF start the *S. cerevisiae* population is in exponential

growth phase (10^6–10^7 colony forming units/ml). In the final step of alcoholic fermentation, over 10% of alcohol, the process is dominated by several *S. cerevisiae* strains. This stage is the most profitable to isolate the fermentative microflora and collect a certain number of genotypes belonging to *S. cerevisiae* species [35, 41].

Performing the grape harvest at ripening time allows to obtain a good degree of yeast biodiversity representing an excellent starting point for the strain selection [32, 43]. The practice of experimental scheme of grape sampling may vary according to the vineyard feature and economic considerations. In optimal situation, the criteria that could be respected have been described by Setati et al. [41]. In detail, it's recommended to:

- Pay attention at any factor which can affect the microbiota community of the vineyard: climate conditions, microclimate (cooler and wetter area may contain a greater population of yeasts), geographical location, microbial vectors, vineyard management (conventional, integrated, organic or biodynamic farming), disease and pests, chemical and pesticides treatment, soil management, and so on [41, 45];

- Collect bunches in proximity of harvest, in order to take the highest *Saccharomyces* spp. biodiversity, also at subspecies level, due to presence of insect and birds at physiological ripeness stage [41];

- A good method to sample is based on the Theory of Sampling (TOS); where a two-dimensional yield is linearised into an elongated one-dimensional lot from which to extract samples at equidistant intervals [41].

As general principles, in the environment and in the vineyard agroecosystem too, yeast populations suffer from spatial and temporal fluctuation, so grape samples should be taken in several locations to gather a sufficient amount of *S. cerevisiae* strains that can be considered for the selection procedure [12, 37, 38]. It should be considered that damaged berries are a source of biodiversity for the sampling of fermentation yeasts [43].

Then, grape bunches should be placed in sterile bags avoiding the contamination with microorganisms unrelated to the sample, and transferred to the laboratory and processed as soon as possible according to the experimental protocol [41].

2.2 *S. cerevisiae* strains isolation

After the harvest of bunches, the spontaneous fermentation must be started, crushing the grapes. In order to avoid the contamination of the cultures, sterile conditions must be ensured by using sterilised or disposable equipment. In this step, di-ammonium phosphate (DAP) can be used as yeast nutrient and SO_2 in the form of potassium metabisulphite can be added to promote the dominance of *S. cerevisiae* strain respect to SO_2-sensitive non-*Saccharomyces*. Alternatively, the process could proceed without any addition of other nutrients or additive, except grape juice. The contact of must with berries skins is essential since the highest yeast concentration is in this compartment. Because of its resistance to osmotic pressure, tolerance to high sucrose concentration and to its efficient fermentation of sugar, *S. cerevisiae* is well adapted to the grape must [12, 42].

Due to the ethanol tolerance of *S. cerevisiae* and to the sensitivity of other yeast species, when the alcoholic fermentation is close to the end (ethanol more than 10% V/V), a sample of fermenting must-wine should be collected to isolate those yeasts that are driving the spontaneous process [12, 42]. Yeast isolation is performed by plating the collected samples on selective laboratory media in controlled conditions.

The dilution of fermenting must or wine at the end of AF is critical to evaluate a reasonable number of colonies in the solid artificial media. However, a compromise with the risk to lose biodiversity with the dilution procedure must be found, so that the sample should represent the yeast population in each vinification. Usually, the sample is diluted until 10^{-5} or 10^{-6} and aliquots of these suspensions are plated. Wallestein Laboratory (WL) agar solid media allowing to differentiate among yeast species on the basis of different colours of the colonies is usually used for yeast growth (**Figure 3**). The incubation temperature must be 24–26° C.

The genotypes loss during the isolation phase, is a problem to deal with during the selection procedure. As the different *S. cerevisiae* strains are morphologically indistinguishable, the colonies must be sampled randomly in plates with 250 colonies maximum. A total of 24–30 colonies for each plate must be sampled and analysed by molecular techniques for species assignation and strain differentiation [46]. Once the isolation and genetic identification phases have been completed, the strains are usually long term stored at −80° C in glycerol 50% V/V to preserve membrane integrity [32, 41, 47] and in slant with YEPD (Yeast Extract Peptone Dextrose) solid agar for short term conservation at 4°C. This procedure has been applied in several studies such as Capece et al. [43], Efstratios et al. [48], Viel et al. [49].

2.3 Genotyping: Molecular biology applied to yeast species identification and *S. cerevisiae* strain characterisation

One of the main goals in microbiology is to obtain a valid identification of microorganisms. Traditionally, before the application of molecular biology techniques, yeasts have been identified by morphological and physiological criteria. These methods are basically labor-intensive, time-consuming, and usually provide doubtful identifications. This is due to similar colony morphology, to the influence of culture conditions on yeast physiology and to the presence of different teleomorphic and anamorphic forms in the same species [50, 51].

The progress in molecular biology allowed to develop fast and efficient methods to identify both species and strains. Methods based on DNA technique,

Figure 3.
Some S. cerevisiae *colonies on Wallestein laboratory (WL) agar medium.*

some of these based on DNA Polymerase Chain Reaction (PCR) proved to be the most effective identification tool. Allozyme patterns, DNA–DNA hybridization, electrophoretic karyotyping, microsatellite analysis, nested-PCR, random amplified polymorphic DNA (RAPD) and mitochondrial DNA restriction analysis are the molecular biology techniques which first contributed to yeast identification [50–58]. As an example, electrophoretic karyotyping is based on the weight analysis of the yeast entire genome according to the species [52]. Other examples of molecular analysis are: insertion site polymorphism of delta elements, simple nucleotide polymorphism (SNP), amplified fragment length polymorphism (AFLP), intron splice sequence amplification, PCR of intron of mitochondrial genes, ribosomal DNA sequencing [12, 54, 57, 59, 60].

Moreover, the genome of *S. cerevisiae* S288C, a model organism in both cell biology and medicine, was entirely sequenced in 1996 and this reference DNA is at the base of the *Saccharomyces* Genome Database (SGD). This achievement facilitates the introduction of new molecular techniques [61, 62].

In this paragraph we will describe more in detail the most relevant techniques for the identification and characterisation of *S. cerevisiae*. RAPD is a PCR based technology in which DNA polymorphism is analysed by amplifying random DNA segments with single primers with an arbitrary nucleotide sequence. A single primer is used to anneal to the genomic DNA at different sites.

Quesada and Cenis in 1995 [53] and Baleiras Couto et al. in 1996 [54] used this method in the taxonomic identification of wine yeast strains both at genera and species level [53, 54]. In 2010, Capece et al. have used a RAPD-PCR with M13 primer to execute a fingerprint on 341 isolates obtaining 130 indigenous strains [43]. This technique can be applied both for interspecific and intraspecific characterisation [55]. The advantage of using RAPD is that it is rapid and easy to assay and there is no need of knowing the DNA sequence, but the main drawback is the low reproducibility.

In 1994, some authors focused the attention on mitochondrial DNA (mtDNA) for fast characterisation of *Saccharomyces sensu stricto complex* [49, 63]. The high polymorphism of this DNA can be highlighted after restriction enzymes digestion (endonucleases: *AluI, DdeI, HinfI, RsaI*). The resulting mtDNA band patterns is species-specific and allows the identification of *S. cerevisiae*, *S. bayanus*, *S. paradoxus*, *S. pastorianus* species [63]. The mtDNA restriction analysis (RFLP-mtDNA) was also applied in many experimentations at strain level due to high degree of intraspecific heterogeneity [42, 47, 64].

For the identification at species level, the main used technique is based on the amplification of the rDNA Internal Transcribe Spacer (ITS) region and subsequent digestion with restriction enzymes. This is a specific type of RFLP also called Amplified Ribosomal DNA Restriction Analysis (ARDRA). The amplified target region includes the conserved gene coding for the 5.8 rRNA subunit and the two flanking non-coding and variable internal transcribed spacers named ITS1 and ITS2 [64, 65].

This method was described by Guillamón et al. in 1998 [64], Granchi et al. [50] and Esteve-Zarzoso et al. in 1999 [51] and is used in oenological yeast species identification still today [50, 51, 64, 65]. According to Guillamón et al. [64], the method is based on a first step of amplification targeting the nuclear rRNA gene region by using primers ITS1 and ITS4. This region includes the coding zone for the RNA ribosomal 5.8S and two non-coding regions at its ends (ITS1 and ITS2) (**Figure 4**). PCR products show a high length variation according to the different species leading to a preliminary discrimination among yeasts after agarose gel electrophoresis. The second step consists in PCR product digestion using three enzymes, endonucleases, *Hinf*I, *Cfo*I and *Hae*III. Each species shows a specific restriction pattern

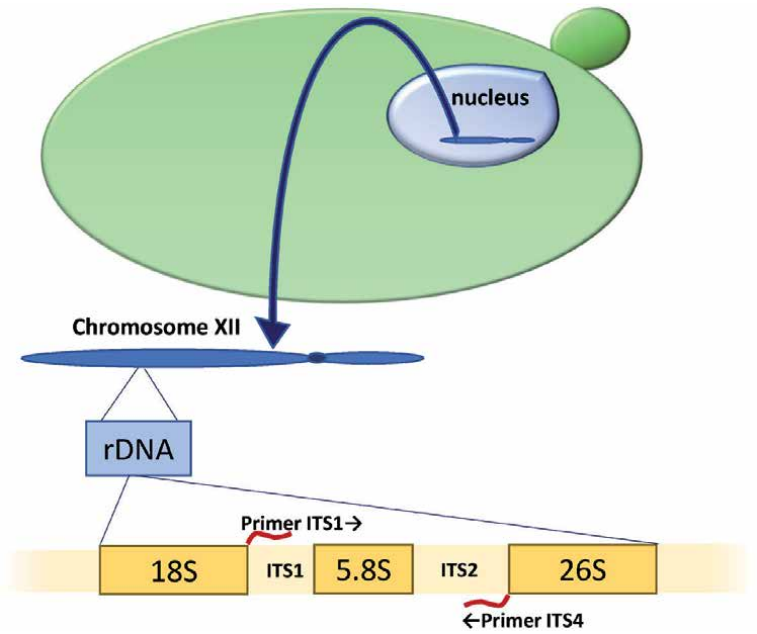

Figure 4.
Nuclear rRNA gene and region of DNA amplification through PCR using primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATA TGC-3′).

according to each endonuclease. So that a discrimination at species level is easily obtained. Thanks to this method it was possible to distinguish with confidence the presence for example of *Hanseniaspora uvarum*, *Candida stellata*, *C. vini*, *S. cerevisiae*, *S. paradoxus*, *S. bayanus*, etc. during spontaneous must fermentation [51, 64, 65]. Similar results have been obtained by Esteve-Zarzoso et al. [51] who analysed 243 different strains belonging to 132 different species, from the Spanish Type Culture Collection (CECT). In the experiment the amplicon digestion has carried out using *HinfI*, *CfoI* and *HaeIII* and other four endonucleases (*AluI*, *TaqI*, *DdeI* and *ScrFI*). This second set of endonouclease was necessary in some particular cases where more restriction patterns were required to get an efficient identification.

In general, this technique is highly reproducible and allows the discrimination of large number of samples.

Focusing on *S. cerevisiae* strain discrimination, inter-delta analysis and microsatellite polymorphism analysis represent useful and easy-to-use molecular tools. Inter-delta regions are some repetitive DNA sequences in *S. cerevisiae* genome, often associated with the transposon Ty1. These regions can be used for the genetic identification of *S. cerevisiae* strains thanks to their different number and location within the species by amplifying these regions with specific primers. Several authors studied inter-delta fingerprinting of *S. cerevisiae* strains and showed that PCR-amplification of DNA delta sequences is a reproducible, strain-specific and simple method that can be successfully applied to monitor strain population dynamics in wine fermentation [47, 66–68].

Microsatellite markers, based on Simple Sequence Repeats (SSRs) scattered throughout the genome [69–73], represent the "gold standard" for this discrimination. Microsatellites are short DNA motifs, 2–6 bases (e.g GATA, GACA, etc.), tandemly repeated five to fifty times (**Table 2**). Their sequence lengths are intra- and interspecific polymorphic across species [56, 69–73]. Moreover, SSRs are characterised by higher mutation rate than the rest of the genome, representing a formidable tool for the genetic differentiation of *S. cerevisiae* strains, as reported by

Locus	SSR Motif	Open Reading Frame Coordinates	Primer sequences (FW: forward; RV: reverse)
ScAAT2	TAA	YBL084c	FW:CAGTCTTATTGCCTTGAACGA **RV:GTCTCCATCCTCCAAACAGCC**
ScAAt3	TAA	YDR160w	FW:TGGGAGGAGGGAAATGGACAG **RV:TTCAGTTACCCGCACAATCTA**
C5	GT	VI-210250/210414	FW:TGACACAATAGCAATGGCCTTCA **RV:GCAAGCGACTAGAACAACAATCACA**
C3	CAA	YGL139w	FW:CTTTTTATTTACGAGCGGGCCAT **RV:AAATCTCATGCCTGTGAGGGGTAT**
C8	TAA	YGL014w	FW:CAGGTCGTTCTAACGTTGGTAAAATG **RV:GCTGTTGCTGTTGGTAGCATTACTGT**
C11	GT	X-518870/519072	FW:TTCCATCATAACCGTCTGGGATT **RV:TGCCTTTTTCTTAGATGGGCTTTC**
YKR072c	GAC	YKR072c	FW:AGATACAGAAGATAAGAACGAAAA **RV:TTATTGATGCTTATCTATTATACC**
SCYOR267c	TGT	YOR267c	FW:TACTAACGTCAACACTGCTGCCAA **RV:GGATCTACTTGCAGTATACGGG**
YKL172w	GAA	YKL172w	FW:CAGGACGCTACCGAAGCTCAAAAG **RV:ACTTTTGGCCAATTTCTCAAGAT**
ScAAT1	TTA	XIII-86902/87140	FW:AAGCGTAAGCAATGGTGTAGATACTT **RV:CAAGCCTCTTCAAGCATGACCTTT**
C4	TAA+ TAG	XV-110701/110935	FW:AGGAGAAAAATGCTGTTTATTCTGACC **RV:TTTTCCTCCGGGACGTGAAATA**

Locus	SSR Motif	Open Reading Frame Coordinates	Primer sequences (FW: forward; RV: reverse)
C9	TAA	YOR156c	FW:AAGGGTTCGTAAACATATAACTGGCA **RV:TATAAGGGAAAAGAGCACGATGGC**
ScAAT5	TAA	XVI-897051/8970210	FW:AGCATAATTGGAGGCAGTAAAGCA **RV:TCTCCGTCTTTTTTGTACTGCGTG**
C6	CA	XVI-485898/485996	FW:GTGGCATCATATCTGTCAATTTTATCAC **RV:CAATCAAGCAAAAGATCGGCCT**
YPL009c	CTT	YPL009c	FW:AACCCATTGACCTCGTTACTATCGT **RV:TTCGATGGCTCTGATAACTCCATTC**
SC8132X (YPL009C)	GAA	XVI-536776/536705	FW: GGTGACTCTAACGGCAGAGTGG **RV: GGATCTACTTGCAGTATACGGG**
SCPTSY7	TTA	XIII-86953/87057	FW: AAAAGCGTAAGCAATGGTGTAGAT **RV: AAATGATGCCAATATTGAAAAGGT**

Table 2.
Some simple sequence repeat motif and primers' origin and sequence for Saccharomyces cerevisiae *typing.*

several papers in last 20 years [46, 49, 56, 69–75]. Hence, they are optimal molecular markers for the strains typing due to their size polymorphism. In general, they are useful for fingerprinting, linkage studies and knowledge on population genetic structure [5, 56, 76].

In 2016, Börlin M. et al. [74] characterised the population structure of more than 653 isolates of *S. cerevisiae* from three French cellars located at less than 10 Km from each other. Using 15 microsatellites loci as molecular markers they observed 503 different genotypes. Hence, based on SSRs analysis and using specific indexes concerning the origin of the three populations it was possible to assess a certain degree of overlapping between genotypes from two of the three cellars and the existence of a local and stable cluster of strains which shared some ancestor over 20 years. The similar composition of the *S. cerevisiae* population structure is explained by a series of events that have repeated over the years. One of these is the proximity of the wineries, which leads to a certain uniformity of the population due to the action of yeast vectors (birds, fruit flies, bees and wasps). And on the other hand, the practice of "pied de cuve", which consists in the inoculation of must with an amount of already fermenting must from a cellar to another. They noted that the SSRs-based method is more robust and sensitive compared to the inter-delta analysis, Pulsed-field Gel Electrophoresis (PFGE) and mtDNA RFPL methods [74].

Rex et al. [76] in 2020 have validated a SSRs molecular markers method for *S. cerevisiae* strain differentiation through PCR-multiplex. The method is based on two multiplex sets of primers of different size targeting polymorphic loci and it was applied on nine well characterised commercial yeasts. A set combines the six primers: ScAAT2, ScAAt3, C5, SCYOR267c, C8, C11, resulting in six different patterns after PCR and gel electrophoresis. The other one combines six other primers: YKL172w, C4, C9, ScAAT5, C6, YPL009c, resulting in five different patterns after the same process. The validation was achieved through the comparison of fragment lengths obtained by capillary sequencing and agarose gel electrophoresis image. The procedure was repeated to characterised 50 strains of *S. cerevisiae* from five different spontaneous fermentations. Through SSRs markers, 21 different new strains were recognised and characterised for their diverse aromatic profile respectively [76].

The strain identification based on SSRs polymorphisms analysis with multiplex PCR application has been used for rapid and low budget procedure too [46]. As an example, Vaudano and Garcia-Moruno [46] performed the typing of 30 commercial wine strains. The discrimination was achieved by performing a multiplex PCR using primers designed on three highly polymorphic loci: SC8132X, YOR267C and SCPTSY7 and subsequent gel electrophoresis and band pattern analysis and comparison.

Then, this analysis was employed in a dominance study between two co-inoculated strain at different temperature of fermentation, 15°C and 20°C. This trial was finalised to control the ability of these *S. cerevisiae* strains in leading the fermentation process.

Methods such as the latter can be used for applicative purpose both in oenology and in wild yeasts selection. In particular, molecular marker supports the screening of the large number of yeasts isolated from natural fermentation [75, 76].

2.4 Phenotype evaluation: technological characterisation, analysis of volatile compounds and sensory evaluation

When different genotypes have been identified, the analysis of the phenotype represented by physiological tests and micro-vinification assay is the following stage of the procedure. The physiological tests are for example:

production of hydrogen sulphide, killer toxin synthesis, SO_2 sensitivity, nitrogen requirement [32, 77].

An interesting test consists in the *in vitro* evaluation of β-glucosidase activity. This enzyme is involved in hydrolysis of monoglucosides with the release of volatile compounds, such as benzenoid/phenylpropanoid, monoterpenes and norisoprenoides, that contribute to aromatic profile. However, β-glucosidase can affect the colour of red wine due to the lysis of anthocyanins compounds with colour alteration or loss; thus the yeast ability to modulate the anthocyanin's colour during AF must be considered in the case of red winemaking [78].

In micro-vinification, the resulting wine is then evaluated through chemical analysis of basic features and volatile compounds [45]. Then, the behaviour of the native strains selected was monitored on a pilot scale in comparison with a known yeast used as control.

An example of this pilot test has been performed in 2019 in Lebanon and aimed to identify the most efficient indigenous starter from three autochthonous *S. cerevisiae* strains previously selected during natural fermentation of Merwah wine (M.6.16, M.10.16, M.4.17). In this study, the fermentation kinetic was evaluated measuring the reduction of the density by using a hydrometer and the residual sugars were analysed by UV–visible spectrophotometry, the dominance of the strains was monitored with Inter-delta-PCR [34].

In any described cases the evaluation of technological characters (**Table 1**) at the end of AF for each indigenous strain considered was always performed, generally using official OIV methods, standards Methods (ISO) or a multiparameter analyser. The more relevant features to be considered are: fermentation trend, ethanol production (%V/V), total acidity (g/l tartaric acid equivalent), volatile acidity (g/l acetic acid equivalent), pH, free and total SO_2 (mg/l), residual sugar (g/l glucose + fructose). For the microbiological stability of wine is essential a residual sugar less than 2 g/l.

Concerning the volatile acidity, it is positive a low-producer yeast, 0.2–0.4 g/l in acetic acid. High producer strains of sulphur compounds are discarded in the selection. SO_2 tolerance is a positive selection criterion [79]. The killer factor is traditionally studied, but its relevance is controversial as it seems that under fermentation conditions it has no influence on sensitive yeast [80].

The evaluation of the phenotype concerns also the wine aromatic profile derived from the secondary metabolism of yeasts. The production of volatile compounds is also affected by the composition of must, in particular depending on the biochemical precursors derived from vine variety. For example, the release of the volatile thiol 4-mercapto-4-methylpentan-2-one (4MMP) from its grape-derived cysteine-bound precursor is carried out by enzymes that possess carbon-sulphur lyase activity and it dependents on yeast [15].

Some volatile compounds belong to the category of higher esters and higher alcohols are shown in **Table 3** [34, 43, 48, 81–88]. In wines, esters can be formed by two different processes: fermentative ones, that involve enzymatic esterification performed by yeast, and storage for long periods that leads to chemical esterification. These two processes can concur in the synthesis of the same ester. The concentration in wine ranges from 10 to 20 mg/l. Higher alcohols are produced by yeasts, both from sugars directly and from grape amino acids through the Ehrlich reaction. They are mostly of fermentative origin and can be found in wines in quantities ranging from 150 to 550 mg/l. The main fermentative higher alcohols, part of the so-called "Fusel oils", are isobutyl alcohol (2-methyl-propan-1-ol) and amyl alcohols (mixture of 2-methyl-butan-1-ol and 3-methyl-butan-1-ol). At concentration lower than 300 mg/l they participate in the aromatic complexity of the wine; at higher concentrations their penetrating odour masks the wine's aromatic finesse.

Volatile Compound	Aroma descriptor	Olfactory threshold	Concentration in Wine	References
Esters				
Ethyl acetate	Fruitiness, varnish	7.5 mg/l*	22.5–63.5 mg/l	Swiegers et al. 2005 [81]
Isoamyl acetate	Banana, pear	0.03 mg/l*	0.1–3.4 mg/l	Swiegers et al. 2005 [81]
Ethyl butanoate	Fruity	0.02 mg/l*	0.01–1.8 mg/l	Swiegers et al. 2005 [81]
Ethyl 3-hydroxybutyrate	Fruity, grapefruit, winy	—	—	
2-Phenyl ethyl acetate	Floral, rose, hyacinth, honey	0.25 mg/l*	0–18.5 mg/l	Swiegers et al. 2005 [81]
Methyl hexanoate	Pineapple	—	—	
Ethyl hexanoate (ethyl caproate)	Green apple, pineapple	0.05 mg/l*	0.03–3.4 mg/l	Swiegers et al. 2005 [81]
Ethyl 2-methylbutanoate	Strawberry	—	—	
Ethyl heptanoate	Grape	—	—	
Ethyl octanoate (ethyl caprylate)	Fruity, floral, wax	0.02 mg/l*	0.05–3.8 mg/l	Swiegers et al. 2005 [81]
Ethyl decanoate (ethyl caprate)	Fruity, apple, soap	0.2 mg/l**	0–2.1 mg/l	Swiegers et al. 2005 [81]
Ethyl dodecanoate (ethyl laurate)	Waxy	—	—	
Ethyl lactate	Buttery, butterscotch	—	—	
Higher alcohols				
Propanol	Alcoholic, pungent, harsh, fermented, weak fusel, musty, yeasty	500 mg/l***	9.0–68 mg/l	Swiegers et al. 2005 [81]
3-Methyl-1-pentanol	Fusel, cognac, wine, cocoa, green, fruity	—	—	
Butanol	Fusel, spiritous	150 mg/l*	0.5–8.5 mg/l	Swiegers et al. 2005 [81]
Isobutanol	Fusel, Ethereal, winey	40 mg/l*	9.0–174 mg/l	Swiegers et al. 2005 [81]
Isoamyl alcohol	Solvent, Varnish, nail polish, ripe fruit, harsh	30 mg/l*	6.0–490 mg/l	Swiegers et al. 2005 [81]
Amyl alcohol	Almond	—	—	
1-Hexanol	Mowed grass, herbaceous, green	4 mg/l***	0.3–12.0 mg/l	Swiegers et al. 2005 [81]
2,3-Butanediol	Fusel, cognac, wine, cocoa, green, fruity	—	—	
2-Phenylethanol	Dried rose, floral	10 mg/l*	4.0–197 mg/l	Swiegers et al. 2005 [81]

Volatile Compound	Aroma descriptor	Olfactory threshold	Concentration in Wine	References
Benzyl alcohol	Jasmine	—	—	
3-(Methylthio)-propanol (Methionol)	Cauliflower	1 mg/l[****]	0.17-2.4 mg/l	Ferreira et al. 2000 [82]
3-Mercapto-1-hexanol	Passion fruit, grapefruit,	$6*10^{-5}$ mg/l[*****]	0-1.28 * 10^{-2} mg/l	Tominaga et al. 1998 [83]

[*] *Aqueous solution 10% ethanol.*
[**] *Synthetic wine.*
[****] *Wine.*
[****] *Red wine.*
[*****] *Aqueous solution 12% ethanol.*

Table 3.
Some volatile compounds from S. cerevisiae *metabolism, respective odour descriptors, olfactory threshold and common concentration in wine.*

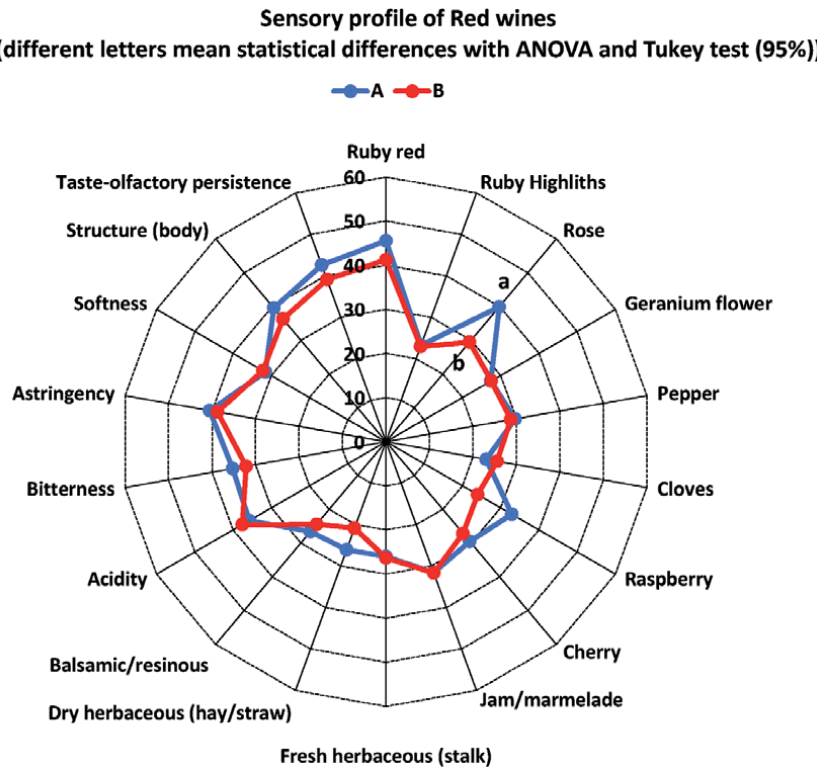

Figure 5.
Comparison of sensory profiles of two (A and B) red wines fermented with two different indigenous strains of S. cerevisiae.

Acetic esters of these alcohols, especially isoamyl acetate, have a banana fragrance that may play a positive role in the aroma of some young red wines (primeur or nouveau) [79].

Usually, the analysis of volatile is performed by gas chromatography equipped with Mass Spectrometer as detector (GC–MS) [43, 48, 81–88].

The last examination at the end of a pilot scale production is the sensory evaluation performed by a panel test. That consist in the personal evaluation of wine

descriptors fulfilled by a group of judges trained in the recognition of organoleptic features (appearance, odour, taste, texture) (ISO 1993). The panel, in short, quantifies the level of descriptors using an intensity scale as required by the ISO 2003 standard b. The sensory session must be performed in standard condition of the room, glasses, temperature, time, so that the environment does not affect the judges [34, 43, 48, 81–88]. An example of sensory analysis results is shown in **Figure 5**. This sensory examination could be useful to predict the consumer appreciation. At the end of this process, all the data obtained by every test must be statistically analysed. The strain or strains which show the best performance and which better meet the enologist's preferences, can be used in an industrial scale assay.

3. Conclusions

In winemaking, the role of yeast is fundamental for a good fermentation process. There is a high biodiversity among the *S. cerevisiae* strains which differently influences the fermentation and the final wine. The choice of the strain is extremely important for the quality and the organoleptic characteristic of wine.

In this chapter a workflow aimed to select indigenous *S. cerevisiae* strains as starter for AF has been described. The main steps are a good sampling in vineyard, the application of rapid but efficient molecular methods, the analysis of the technological features and the final sensory properties.

In consideration of the increasing appreciation by consumers of wines connoted by organoleptic complexity also linking with the territory of origin, the selection of indigenous *S. cerevisiae* strains represents a valid and safe scientific approach aimed at the production of wines with a typical character (terroir).

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Laura Pulcini[1,2*], Elisa Gamalero[2], Antonella Costantini[1], Enrico Tommaso Vaudano[1], Christos Tsolakis[1] and Emilia Garcia-Moruno[1]

1 Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria – Centro di Ricerca Viticoltura ed Enologia (CREA-VE), Asti, Italy

2 Dipartimento di Scienze e Innovazione Tecnologica, Università degli Studi del Piemonte Orientale, Alessandria, Italy

*Address all correspondence to: laura.pulcini@crea.gov.it

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Chapter 9

Genetically Modified Yeasts in Wine Biotechnology

Cecilia Picazo, Víctor Garrigós, Emilia Matallana and Agustín Aranda

Abstract

Modern enology relies on the use of selected yeasts, both *Saccharomyces* and non-conventional, as starters to achieve reliable fermentations. That allows the selection of the right strain for each process and also the improvement of such strain, by traditional methods or approaches involving genetic manipulation. Genetic engineering allows deletion, overexpression and point mutation of endogenous yeast genes with known interesting features in winemaking and the introduction of foreign and novel activities. Besides, it is a powerful tool to understand the molecular mechanisms behind the desirable traits of a good wine strain, as those directed mutations reveal phenotypes of interest. The genetic editing technology called CRISPR-Cas9 allows a fast, easy and non-invasive manipulation of industrial strains that renders cells with no traces of foreign genetic material. Genetic manipulation of non-*Saccharomyces* wine yeasts has been less common, but those new technologies together with the increasing knowledge on the genome of such strains opens a promising field of yeast improvement.

Keywords: wine, *Saccharomyces cerevisiae*, non-*Saccharomyces* yeasts, genetically modified organisms, gene editing

1. Introduction

Wine production is a process that happening since the antiquity. For more than 7000 years there has been a continuing evolution in grape juice fermentation and wine production. Humans have used yeasts for wine production without any knowledge about them. Yeast cells were observed for the first time in a microscope in 1680 by Antoine Van Leeuwenhoek. Between 1850 and 1875, Louis Pasteur the role of yeast in alcoholic fermentation for the first time [1]. Grape juice fermentation is a complex microbiological process with a lot of microorganism interactions (yeast, bacteria, filamentous fungi) [2]. *Saccharomyces cerevisiae* has been identifying as the main microorganism responsible of the grape juice fermentation and the bacteria *Oenococcus oeni* as the one for malolactic fermentation that is important for some wines. But in the grape surface there are a lot of species of yeast, while *S. cerevisiae* is hardly found in the vineyard, although is a common resident in winery environments. Non-*Saccharomyces* yeasts contribute to the organoleptic complexity of wine, but are displaced by *Saccharomyces* species that are strong fermenters and are highly tolerant to ethanol [3]. Modern enology relies on the use of starters, generally in the form of active dry yeast (ADY). Select the yeast that you are going to use is

important to have a fast and complete fermentation, decrease lag phase and have a reproducible parameter in the final product [4]. Those starters have been isolated from many environments for their good performance, but they can be further improved by human action by different means.

In this chapter, we focused on the study of the improvement of wine yeast for wine production by recombinant technologies that produce Genetically Modified Organisms (GMOs). That allows a better understanding of the molecular processes relevant for wine yeasts too. We will describe the aspects that have been targeted for improvement, the new technologies of gene editing and synthetic biology and the potential use of these technologies on non-conventional yeasts.

2. Improving relevant winemaking aspects by genetic manipulation

The traditional ways of genetically manipulating yeast include gene deletion, gene overexpression under the control of heterologous promoters or the introduction of foreign genes [5]. The latter can be done using plasmids (both single or multicopy) or seeking a more stable chromosomal integration. Many different systems for modifying chromosome sequences inside cells have been created. A PCR-based gene targeting approach, that uses exogenous DNA introduced into the cell through various transformation methods, has become one of the most widely used. Selectable markers, sometimes involving antibiotic resistance are needed for validation and maintenance of integrated sequences. To eliminate those markers, scientists used a marker recycling approach that takes advantage of site-specific recombinase technologies. loxP-mediated Cre recombinase is a good example of this method [6]. Many characteristics of wine strains of *S. cerevisiae* can be improved by gene manipulation. We will focus on stress tolerance, nutrient optimization, sensory improvement and health enhancement.

2.1 Improving stress tolerance

S. cerevisiae must deal with different stress conditions, osmotic stress due to the high levels of sugars, oxidative stress, low nitrogen levels and high levels of ethanol among others. These stresses can produce problems in the wine fermentation process [7]. One way to solve these problems is to use engineering yeast strains that can grow better in these conditions.

The main component in the grape juice is monosaccharides (glucose + fructose) and their total concentration vary between 170 and 220 g/L [2] but can be up to 340 g/L. This extremes levels of sugars can inhibit yeast growth because of the osmotic pressure, that is called hyperosmotic stress. High Osmolarity Glycerol response (HOG) is the pathway that are regulated the response against osmotic stress, inducing the gene expression for glycerol production (*GPD1* and *GPD2*) and for glycerol uptake (*STL1*) [8]. Deletion of Stl1 (glycerol symporter) has a slower growth in ice wine juice and elevate glycerol and acetic acid production, so these genes could be a target to improve these conditions [9].

Aerobic organisms depend on oxygen in cellular respiration but at high concentrations its oxidant power produces cytotoxic compounds called reactive oxygen species (ROS) that are unstable oxygen species with unpaired electrons that if they are not remove from the cell can damage macromolecules as DNA, proteins and lipids. It is during the active dry yeast production (ADY) where the yeast is in a higher oxidative stress condition. For example, oxidative stress-related genes (as thioredoxines, glutaredoxins and peroxiredoxins) are induced during this process [10]. Overexpression of the cytosolic thioredoxin 2 gene, *TRX2*, leads a wine yeast

increase biomass production [11]. This ADY process cause an internal oxidative stress and there are molecules and enzymes that helps to reduce the oxidative stress as glutathione (GSH), trehalose, catalase, superoxide dismutase and glutathione reductase [12]. For example, deletion of the main cytosolic peroxiredoxin, Tsa1, in the industrial wine yeast L2056 increase trehalose and glycogen accumulation playing a role in the regulation of metabolic reactions that are important for the final product [13]. Moreover, overexpression of superoxide dismutase 1 and 2 (*SOD1* and *SOD2*) and *HSP12* (a plasma membrane protein involved in maintaining membrane organization) genes improves vellum formation and cell viability in three strains of Sherry flor yeast, and improve in the specific activities and higher levels of GSH peroxidase and glutathione reductase activities and higher intracellular concentrations of GSH and lower peroxidized lipid concentration [14]. Moreover, an indigenous strain of *S. cerevisiae* called RIA with the insertion thought homologs recombination of ilv2Δ::GSH1-*CUP1* improves glutathione production (19%) with the same fermentation capacity than the wild type [15].

At the end of the fermentation process, there are high levels of ethanol (11–14%). The toxicity of ethanol inhibited glucose and amino acid uptake because ethanol damage cell membranes [2, 16]. Overexpression of *TPS1* (synthase subunit of trehalase-6-P synthase/phosphatase complex) and deletion of *NTH1* (neutral trehalose) increase ethanol tolerance [17]. Besides, overexpression of *GSY2* (Glycogen synthase) and *NTH1* increased respectively glycogen and trehalose levels that are important for the fermentative capacity [18]. Global transcription machinery engineering (gTME) is a technique that alter key proteins to regulate the global transcriptome by error-prone polymerase chain reaction (epPCR) mutations. With this technique, a *SPT15* (TATA binding protein) mutagenesis strains was constructed with a higher ethanol tolerance [19] and it was found that the mutant of the *SPT8* (SAGA complex) gave 8.9% higher ethanol tolerance [20]. Direct evolution method was performed to engineer RNA polymerase II (RNAPII) subunit 7 (which plays a central role in mRNAs synthesis) in the yeast strain (M1) that improved ethanol titer and improved other stress as osmotolerance [21].

Some species of *Saccharomyces* genus have shown better adaptation at low temperatures than *cerevisiae*, which was the case of cryotolerant yeast *S. uvarum* and *S. kudriavzevii*. This better cold adaptation is because the higher amount of proteins related with translation (more ribosomes proteins in psychrotolerant strains) and the importance of the oxidative stress response in the adaptation of cold fermentation (mutants in *AHP1*, *MUP1* and *URM1* has a strongly impaired low-temperature growth) [22, 23]. Recently, Ying Su *et al* noticed that the hybrids low nitrogen-demanding cryotolerant *S. eubayanus* and *S uvarum* conferred better fermentations rates under low temperature or low-nitrogen conditions [24].

2.2 Nutrient usage and fermentation performance

The right use of metabolites is key for a successful fermentation. One of the most important steps in the fermentation process is the hexose uptake. Overexpression of fructose/H^+ symporter *FSY1* from *S. pastorianus* results in improve glucose and fructose uptake during wine fermentation [25]. Moreover, using a null hexose transporter mutant *HXT1* to *HXT7* of *S. cerevisiae* (KOY.TM6*P) and overexpression of chimeric *HXT1-HXT7* gene in this strain showed that there is a decreased ethanol production and increased biomass under high glucose concentration [8]. The first step of the glycolysis is depend on the role of cytosolic thioredoxins 1 and 2. The double mutant of these thioredoxins in the haploid wine yeast C9 (derived from commercial strain L2056) has a problem in the use of the sugars at the levels of the hexokinase 1 and 2 and in the glucokinase 1 that produce a slow fermentation [26].

One of the most important nutrients in the grape juice is the nitrogen and it could be a limiting nutrient for the growth of yeast because low levels of nitrogen can stop the fermentation when the sugars are still remained in the medium. *S. cerevisiae* cannot assimilate inorganic nitrogen nor polypeptides and proteins, so its grow depend on ammonium and free amino acids, called YAN (Yeast Assimilable Nitrogen). Concentrations below 140 mg/L of YAN in a normal sugar concentration, can produce negative effect in the fermentation process and nitrogen depletion irreversibly arrest hexose transport. One way to improve the nitrogen assimilation is through deletion of *URE2* repressor of alternative nitrogen sources as prolines. It controls the *PUT1*-encoded proline oxidase and *PUT2*-encoded pyrroline-5-carboxylate dehydrogenase to create yeast that can efficiently assimilate the abundant supply of proline and arginine in grape juice [25, 27]. *MFA2* deletion (encoding mating factor-a) is another way to improve the fermentation efficiency under nitrogen limitation (75 mg/L). They used a deletion in the haploid wine yeast AWRI1631 under microvinification conditions [28]. Another work by Jin Zahng using a transposon library in wine yeast, selected five candidate genes to efficiently complete a model of oenological fermentation with limited nitrogen availability. They did the gene disruptions in the haploid wine yeast C911D where they found that the deletion of *ECM33* (GPI-anchored protein involved in efficient glucose uptake) resulted in the shortest fermentation (up to 31%) in grape juice and there were no differences in the nitrogen utilization, cell viability or biomass with the parental strain. This mutant has an up-regulation in the cell way integrity regulated genes [29].

2.3 Increasing the quality of the wine

Understanding wine flavor compound composition is a key to improve the final product. Yeast metabolism during wine fermentation produce ethanol and secondary metabolites that are important for the wine. The generation of wine yeast able to produce wines with reduced ethanol concentrations while retaining harmonious balance between the level of alcohol, acidity, sweetness, and other sensory qualities has been the focus of extensive research. The main idea is to divert partially the carbon metabolism from the formation of ethanol to glycerol, but it is difficult to do it without a significant impact on wine quality, as acetic acid rises [30]. For example, overexpression of the main glycerol producing enzyme *GPD1* (NAD-dependent glycerol-3-phosphate dehydrogenase) together with the deletion of *ALD6* (aldehyde dehydrogenase) is able to decrease acetic acid production in the strain AWRI2531 and produce a fermentation with 15–20% less ethanol and more glycerol [31]. Reduction of 7.4% of ethanol without negative consequences was possible through the partial deletion of *PDC2* (transcription factor required for expression of the two isoforms of pyruvate decarboxylase *PDC1* and *PDC5*) [32]. Overexpression of *TPS1* (trehalose synthase gene) produce a 10% ethanol decrease [33]. NADH oxidase was expressed in *S. cerevisiae* so the NADH pool was reduce getting a 15% lower of ethanol but the redox reactions and grow was affected [34]. Decreases in ethanol levels was carry on by expression of *GOX1* (glucose oxidase gene) from *Aspergiullus niger* [35]. Alternative, deletion of TORC1 pathway kinase *SCH9* in the haploid wine yeast C9, increase glycerol production during wine making conditions [36].

The most significant effect on the aroma of wine are acetate esters, ethyl acetate (fruity and tart aromas), 2-phenylethyl acetate (honey, rose) and isoamyl acetate (banana flavor) [37]. Increase these compounds in the wine is important to get a good final product. Overexpression of *ATF1* (alcohol acetyltransferase) got a significant increase in acetate ester production. Moreover, deletion of *ATF1* and *ATF2* abolished the formation of isoamylacetate but still produces ethyl acetate and

overexpression of esterase (*IAH1*) decrease significantly concentration of ethyl acetate and isoamyl acetate among others [38, 39].

Terpenoids or isoprenoids are naturally compounds which are involved in the fragrance and aroma of flowers and fruits. One way to improve the production of these positive compounds in the wine is using genes from species that produce this aroma. For example, using S-linalool synthase (*LIS*) from *Clarkia breweri* in *S. cerevisae* produce a novo production of linalool in wine about 19 μg/L [40]. Through the expression of the *Ocimun basilicum* (sweet basil) geraniol synthase (*GES*) gene in the industrial wine yeast T73, Pardo *et al.* got an recombinant yeast which excreted geraniol de novo at an amount 750 μg/L that was further metabolized in other interested monoterpenoids and esters as citronellol, linalool, nerol, citronellyl acetate and geranyl acetate [41]. Expression and secretion of the *Aspergillus awamori* α-L-arabinofuranoside in combination with either β-glucosidase from *Saccharomycopsis fibuligera* or from *Aspergiluus kawachii* in the industrial yeast VIN13 has higher concentrations of monoterpenoids and improve sensory characteristics [42].

Other volatile sulfur compound is hydrogen sulfur, H_2S, that has an undesirable 'sulfurous', 'rotten egg'-like off flavor even at low concentrations (1 μg/L) that it is a significant problem for the global wine industry. Reduced H_2S amount in the wine it is another improvement that can has beneficial effects for the wine. Specific site directed mutation in both *MET10* and *MET5* genes (α and β subunits of sulfite reductase enzyme) reduced by 50–99% the H_2S production depending on the strain [43]. Using the strain UCD932 a strain producing little or no detectable H_2S during wine fermentation was constructed and identified the allele of *MET10* (*MET10–932*) as a responsible. Replacing the *MET10* allele of high- H_2S producing strain with *MET10–932* prevented H_2S formation [44].

2.4 Improving human health

Yeast metabolism can be diverted to produce compounds that has specific influence in human health. This section will focus on two beneficial compounds for human health (resveratrol and hydroxytyrosol) and one potentially dangerous, ethyl carbamate.

Grape juice has a lot of polyphenols, one of them, resveratrol is a stress metabolite produced by *Vitis vinifera* grape vines and it is a potent antioxidant with multiple beneficial effects. Red wines contain a much higher resveratrol concentration than white wine, due to skin contact during fermentation [45]. In plants, resveratrol synthesis is from malonyl-CoA and *p*-coumaroyl-CoA by the resveratrol synthase. But *in S. cerevisiae* coenzyme -A ligase is absent, and it is necessary for the last steps of the resveratrol synthesis. In 2003, Becker *et al.* by co-expressing the coenzyme-A ligase gene (*4CL216*) from a hybrid polar and the grapevine resveratrol synthase gene (*vstl1*) resveratrol production was successfully for the first time. Introduction of 4 heterologous genes (phenylalanine ammonia lyase gene from *Rhodosporidium toruloides*, the cinnamic acid 4-hydroxylase and 4-coumarate coenzyme A ligase genes both from *Arabidopsis thaliana*, and the stilbene synthetase gene from *Arachis hypogaea*), overexpression of acetyl-CoA carboxylase gene (*ACC1*) and addition of tyrosine to the medium produced an increase in concentration of resveratrol up to 5.8 mg/L in *S. cerevisiae* laboratory W303-1A strain [46]. Moreover, two expression vector carrying 4-coumarate coenzyme A ligase gene (4CL) from *Arabidopsis thaliana* and resveratrol synthase gene (RS) from *Vitis vitifera* were introduced in the industrial yeast EC1118 [47]. This strain produced 8.25 mg/L of resveratrol. Indeed, resveratrol was produced with fed-batch fermentation directly from glucose (416.65 mg/L) and from ethanol (531.41 mg/L) [48]. With an optimization of the same strategy with the electron transfer to the cytochrome P450 monooxygenase,

800 mg/L of resveratrol was obtained [49]. Recently, a co-culture platform with two different species was used to produced 36 mg/L [50]. *Escherichia coli* excrete *p*-coumaric acid into the media and *S. cerevisiae* with an inactivation-resistant version of acetyl-CoA carboxylase ($ACC1^{S659A,S1157A}$) that modulate constitutively the expression of 4-coumarate-CoA ligase from *Arabidopsis thaliana* (4CL) and resveratrol synthase from *Vitis vinifera* (STS) to produce resveratrol.

Another polyphenol that has a strong antioxidant capacity is hydroxytyrosol (HT). It is found it in extra virgin olive oil, less in wine (with a range between 0.28–9.6 mg/L). In yeast, tyrosol is synthesized from tyrosine through the well-established Ehrlich pathway. In bacteria, there are some ways to produce hydroxytyrosol using yeast genes. For example, co-expression of yeast *ARO8* and *ARO10* genes for an important accumulation of tyrosol when was added in the media. Moreover, co-expression of yeast *ARO10* and *ADH6* and the overexpression of the native aromatic hydroxylase complex HpaBC produce important amounts (647 mg/L) of HT in *E. coli* [51]. Recently, HT production (4 mg/L) was possible with the introduction of the *E. coli* hydroxylase HpaBC complex components (hpaB and hpaC) in laboratory BY4743 yeast strain and with the addition of tyrosol to the media [52].

Ethyl Carbamate (EC) is a toxic present in wines. During wine fermentation, *S. cerevisiae* metabolizes arginine (one of the major amino acid in grape juice) using arginase *CAR1* to ornithine and urea, but this urea is not fully metabolizing and is secreted. Urea degradation is an energy-dependent two-step process catalyzed by urea amidolyase (*DUR1*, *2* genes). The urea that is secreted by yeast to the media can react with the ethanol of the wine to form ethyl carbamate that is classified as probably carcinogenic for humans. With the overexpression of *DUR1* and *DUR2* under *PGK1* promoter, 89.1% less of EC was developed in Chardonnay wine [53]. Deletion of *CAR1* in the YZ22 strain blocked urea secretion and there is a reduction of EC production [54]. This fermentation results showed that the content of urea and EC in wine decreased by 77.89% and 73.78% respectively and no differences were detected in growth and fermentation parameters with the parental strain.

3. New technologies in genetic engineering

The traditional methods of genetic manipulation are time-consuming when dealing with industrial strains, as they usually have multiple copies for each gene. Gene editing by using the CRISPR-Cas9 technology is faster to cause multiple gene deletions and introducing punctual changes. New tools as genome editing and genome synthesis are building up a new era for the synthetic biology. Their application for yeasts of biotechnological interest will change the paradigm in the ways we approach the use of those microorganisms for a particular task, as their abilities can be tailored from the beginning to the end.

3.1 Wine yeasts genome editing by CRISPR-Cas9

Industrial yeast strains are usually diploid or polyploidy with a more complex genetic background than the well-studied haploid laboratory strains. Using a traditional PCR-based technique for the genetic manipulation of industrial strains is normally very time consuming, laborious and often even impossible [55]. In recent years, the development of an alternative genome editing approach, Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (CRISPR–Cas9) system, can help to solve the problem [55, 56].

At first, CRISPR-Cas system was discovered to provide an immunological weapon for bacteria and archaea against the attack by viruses (bacteriophages) or

invading mobile genetic elements [57, 58]. The CRISPR system from *Streptococcus pyogenes* has been well characterized and it is still the most widely used in yeast genetic engineering. Two elements are necessary for the correct operation of the CRISPR-Cas system. The Cas9, a 160 kilodalton protein, is a RNA-mediated endonuclease that recognizes a 3-nucleotide protospacer adjacent motif (PAM), NGG (where N is any nucleotide, followed by two guanines (G)), and makes double-stranded breaks (DSBs) between the third and fourth nucleotides upstream to the PAM site. Another key component is a single guide RNA (sgRNA) that guides Cas9 to target sites. The sgRNA derives from a duplex of two RNA molecules: a CRISPR targeting RNA (crRNA), which is complementary to the target, and a trans-activating CRISPR RNA (tracrRNA). The first 20 base pairs at 5′ end of crRNA binds to the complementary genomic target, and PAM site must be found immediately at 3′ end of the desired locus in genome [59]. The sgRNA has a concrete secondary structure to recruit Cas9 to establish a functional complex. Following the guide of sgRNA, Cas9 target the genome specific sequence with PAM and cut double-strand DNA [60]. DSB must be repaired by cells via non-homologous end-joining (NHEJ) or homologous recombination (HR). Normally, NHEJ repair is considered to generate small nucleotide insertions or deletions, and HR is used for precise modifications with the existence of donor DNA (**Figure 1**).

CRISPR-Cas9 genome-editing technology was first applied in *S. cerevisiae* in 2013 [56]. Vigentini and co-authors successfully established the CRISPR-Cas9 system in commercial wine strains EC1118 and AWRI1796. In this study, *CAN1* gene encoding for an arginine permease was deleted, in order to generate strains with reduced urea production [61] (see above about EC). The resulting *can1*Δ mutants were characterized by decreased urea production (18 and 35.5% compared to EC1118 and AWRI1796, respectively) under micro-winemaking conditions, in Chardonnay and Cabernet Sauvignon grape musts. Recently, Wu et al. [62] use CRISPR-Cas9 system for over-expressing the *DUR3* gene in a previously engineered rice wine strain with *CAR1* gene disrupted and *DUR1,2* genes over-expressed [63]. *CAR1* encodes an arginase responsible for the arginine cleavage generating urea. Urea can be hydrolysed into NH_3 and CO_2 by urea amidolyase (encoded by *DUR1,2*), and *DUR3* encodes a transporter that transfers urea from the fermentation broth to yeast cells when nitrogen source is insufficient. A laboratory fermentation experiment of Chinese rice wine shows that the CRISPR-Cas9 engineered strain reduces urea and EC concentrations by 92% and 85%, respectively, compared with those of the original strain (N85).

In another work, a polygenic analysis combined with CRISPR-Cas9-mediated allele exchange reveals novel *S. cerevisiae* genes involved in the production of 2-phenylethyl acetate (PEA). PEA is a desirable flavor compound that provides alcoholic beverages a rose and honey aromas. With the mentioned approach, unique alleles of the *FAS2* gene (encodes de α subunit of fatty acid synthase) and a mutant allele of *TOR1* (growth regulator in response to nitrogen sources) were identified to be responsible for high PEA production. Then, using CRISPR-Cas9, wild type alleles were replaced with mutant ones in commercial wine strains. PEA production in these yeasts increased by 70% [64].

In a recent study, Walker and co-authors [65] used CRISPR-Cas9 system to introduce selected mutations in *SUL1* and *SUL2* genes in wine strains EC1118. These genes encoded two high-affinity sulfate transporters. Under nitrogen limitation, sulfate contributes to hydrogen sulfide (H_2S) production, a common wine fault with a rotten-egg odor. The introduced mutations affect protein-structure function of Sul1 and Sul2 and shown to reduce H_2S accumulation during fermentation in Riesling juice or a chemical defined grape juice.

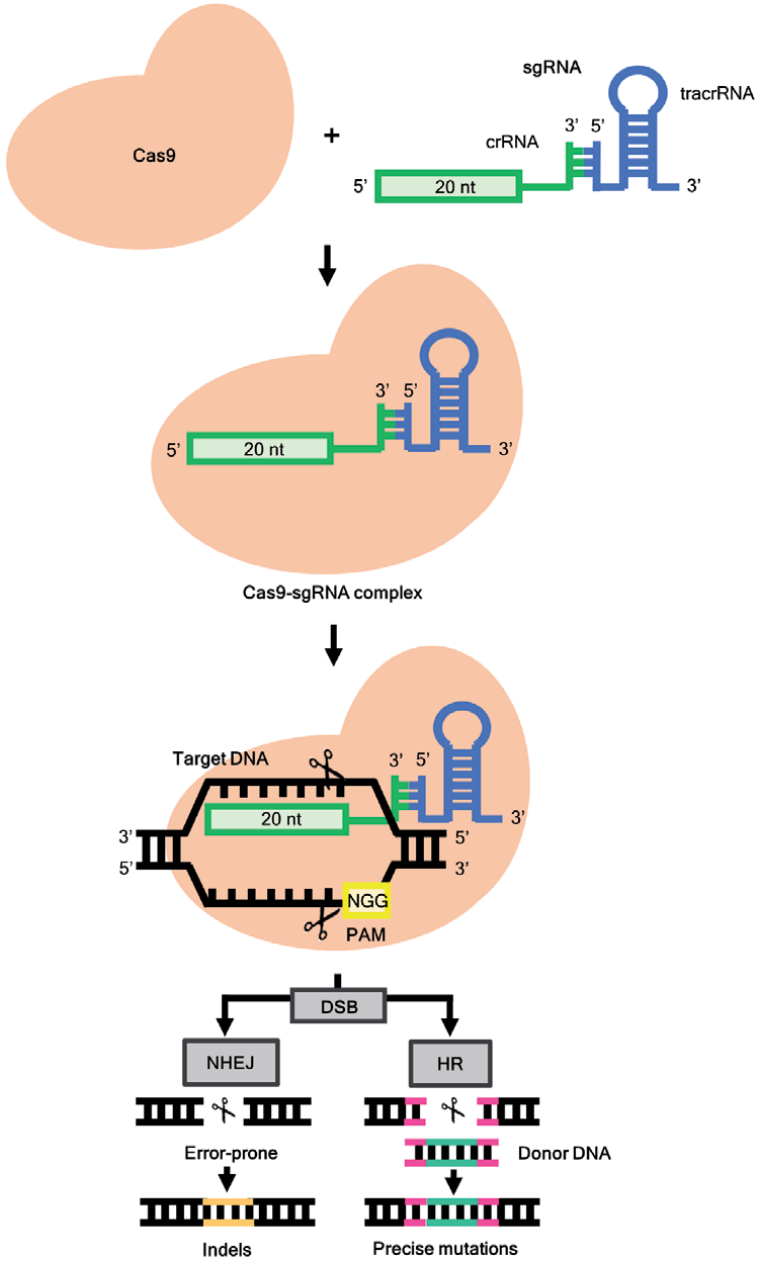

Figure 1.
Overview of the CRISPR-Cas9 system. The Cas9 interacts with sgRNA and form a complex. The Cas9-sgRNA complex binds to the target DNA sequence upstream of PAM site. The Cas9 protein cleaves DNA sequence complementary to the 20 bp guide sequence producing a double-strand break (DSB). After the nuclease cuts the DNA can be repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR).

In *S. cerevisiae*, glycerol is a key polyol that reduces osmotic stress and controls intracellular redox balance. Muysson et al. [66] established a CRISPR-Cas9-based genome-editing approach to investigate the Stl1p (a H^+/glycerol symporter) role in ice wine fermentations. In this study, *STL1* gene was deleted in *S. cerevisiae* K1-V1116 strain. During ice wine fermentation, the *stl1*Δ mutant presents increased glycerol and acetic acid production compared to the original strain, suggesting that Stl1 plays an important role in these conditions. In a study carried out by van Wyk et al. [67], CRISPR-Cas9 system was used to increase glycerol and ester

production in the AWRI1631 wine yeast strain. First, two newly strains were created, one that overexpressed *GPD1* and the other that overexpressed *ATF2*. *GPD1* encodes a glycerol-phosphate dehydrogenase involved in glycerol formation; and *ATF2* encodes an alcohol acetyltransferase which promotes condensation between alcohols and acetyl-CoA resulting in more acetate esters produced, important for flavor in fermented beverages. Mating these engineered strains, the authors obtained a new strain that overexpressed *GPD1* and *ATF1* genes. Riesling wine from the resulting strain showed increased glycerol and acetate ester levels compared to the parental strain.

Vallejo and co-authors [68] described recently that nutrient signaling pathway genetic manipulation can be a good target of yeast performance improvement during winemaking. Using CRISPR-Cas9 system in commercial wine strain EC1118, *PDE2* gene encoding for a phosphodiesterase was deleted. Pde2 is a cAMP degrading enzyme whose deletion increases cAMP-dependent protein kinase A (PKA) activity. The resulting *pde2*Δ mutant showed increased fermentation speed compared to EC1118, in red grape juice. The results suggest that Pde2p inactivation is a way to increase fermentative performance.

3.2 Synthetic genome engineering

Synthetic biology seeks to standardize and modularize the design and engineering of organisms to achieve novel functions, or to construct genomes or even organisms from the ground up using rational laboratory procedures or automation [69]. Synthetic biology is regarded as the most exciting interdisciplinary science of the twenty-first century, with applications in yeast biotechnology and strain development, among other things. Given yeast's importance in the fermentation industry as well as its role as an experimental research model organism in the advancement of Synthetic Biology, the wine industry will be impacted by the outcomes of this field. Synthetic Biology techniques are already being applied to the production of better wine yeast strains [70, 71].

In 1996, the 14 Mb genome of a haploid laboratory strain (S288c) of *S. cerevisiae* was sequenced for the first time, revealing that its 16 chromosomes encode 6000 genes, of which 5000 are non-essential. In 2009, the first synthetic yeast genome project (Sc. 2.0 project) was launched to redesign and chemically synthesize a slightly modified version of the *S. cerevisiae* S288c strain genome. This project allows to find answers to a broad range of questions about fundamental properties of chromosomes, genome organization, gene content, RNA splicing mechanism, the role of small RNAs in yeast biology, the distinction between prokaryotes and eukaryotes, and genome structure and evolution [72]. The Sc2.0 genome was designed to contain specific base substitutions inside some of the ORFs to accommodate desirable enzyme recognition sites or deletions of undesirable enzyme recognition sites. All TAG stop codons were recoded to TAA to free up one codon for future inclusion of unusual amino acids; all repetitive and dispensable sequences were omitted; and all tRNA genes were relocated to a novel neochromosome.

In 2011, the first step toward building the ultimate yeast genome was taken with the construction of synthetic chromosome arms [73]. In 2014, *S. cerevisiae* became the first eukaryotic cell to be equipped with a fully functional synthetic chromosome, the chromosome 3 [74]. In 2017, six redesigned yeast chromosomes were completed [72]. In 2018, 16 natural chromosomes of *S. cerevisiae* were successfully fused into a single chromosome, like in prokaryotic cells, and the artificial *S. cerevisiae* still has normal cellular functions [75]. These works blur the lines between natural and artificial life, pointing to a near-future for custom-designed yeast to fulfill all the customers' needs.

Wine yeast strain development is well positioned to benefit from technological advances made with the genetic and genome engineering of non-wine strains of *S. cerevisiae*. For example, the first "synthetically engineered" wine yeast reveals a whiff of raspberries in an experimental Chardonnay wine. *S. cerevisiae* AWRI1631 wine strain was equipped with a biosynthetic pathway, including four separate enzymatic activities, to produce the highly desirable raspberry ketone (4-(4-hydroxyphenyl)butan-2-one) [76].

4. Genetic manipulation of non-*Saccharomyces* yeasts

Despite the increasing relevance of non-conventional yeast in modern enology, there are few examples and tools of genetic manipulation for those yeasts. The targets of modification are shared with *S. cerevisiae* strains and usually are devoted to an organoleptic improvement. Recently, Badura and co-authors [77] developed a tool for the genetic modification of *Hanseniaspora uvarum*. In the past, *Hanseniaspora* populations have been regarded to be spoilage yeasts due to some strains produce large quantities of acetaldehyde, acetic acid, and ethyl acetate. However, *Hanseniaspora* wine strains have oenological benefits such as lower final ethanol levels and higher acetate and ethyl ester concentrations. In this study, authors used a traditional PCR-based technique for the disruption of the *HuATF1,* which encodes a putative alcohol acetyltransferase involved in acetate ester formation. This approach introduces the first steps in the development of gene modification tools of this yeast.

Some *Kluyveromyces marxianus* strains are able to ferment sugars in high temperature environments (up to 45°C) including grape juice [78]. This yeast is also in some commercial preparations of yeast to contribute flavor complexity. In a study published in 2014, *K. marxianus* BY25569 strain was evolved and genetically engineered for overproduction of 2-phenylethanol (2-PE) from glucose [79]. 2-PE confers "rose" and "floral" scents, almost non-existent but interesting in winemaking. *Kluyveromyces lactis* is a kind of non-*Saccharomyces* yeast that aims to solve the problem of low total acid and high pH in wine, due to its high lactate production. In this direction, *K. lactic* was genetically modified by introducing a heterologous L-lactate dehydrogenase gene (LDH) and deleting pyruvate decarboxylase gene KlPDC1 and/or the pyruvate dehydrogenase (PDH) E1 subunit gene [80, 81]. With these modifications, the central carbon flux of *K. lactis* was diverged from the production of ethanol to enhance lactate production. *K. lactis* was also metabolically engineered for L-ascorbic acid (vitamin C) production [82]. On the palate, the wines with added ascorbic acid were perceived as less oxidized, less ripe and fresher. To achieve this aim, GDP mannose 3,5-epimerase (GME), GDP-L-galactose phosphorylase (VTC2), and L-galactose-1-phosphate phosphatase (VTC4) from *A. thaliana* were introduced in *K. lactis* CBS2359 strain.

Pichia pastoris has been described as one of the most popular and standard tools for the production of recombinant protein in molecular biology [83]. This fact can be exploited in the wine production field. For example, The EPG1–2 gene, which codes for an endopolygalacturonase in *K. marxianus* CECT1043, has been expressed in *P. pastoris* X33 strain [84]. The use of this endopolygalacturonase improves Albariño wine aroma, providing an increase of citric, balsamic, spicy and above all floral (violet and rose) aromas [85].

CRISPR-based genome-editing approaches have also been applied in many non-conventional yeasts. However, due to non-*Saccharomyces* species had been considered spoilage yeasts in wine fermentations, and CRISPR in wine yeasts still falls under the definition of GMOs of the European regulations, less progress has been

made in the field of fermented foods and beverages. Therefore, in non-conventional yeasts CRISPR-Cas9 system has been applied mainly in the production of biofuels, chemicals, nutraceuticals, enzymes or recombinant proteins [86, 87]. In *Pichia pastoris* (syn. *Komagataella phaffii*), CRISPR-Cas9 system has been applied to improve its efficiency for the production off high-value pharmaceuticals [88]; in *Ogataea polymorpha*, a thermotolerant methylotrophic yeast, for the production of bioethanol [89] or for the introduction of all the genes necessary for the biosynthesis of resveratrol [90]. For biofuels and chemicals production in *Issatchenkia orientalis* [91]; in *Kluyveromyces marxianus* for its use as cell factory [92, 93]; in *Kluyveromyces marxianus* for the production of recombinant proteins [94, 95], or for integrating a synthetic muconic acid pathway [96]; in *Schizosaccharomyces pombe* [97]; in *Candida* species for the production of xylonic acid and ethanol [98] or for biosynthesis of β-carotene and its derivatives [99]; and in *Yarrowia lipolytica* for the production of renewable chemicals and enzymes for fuel, feed, oleochemical, nutraceutical and pharmaceutical applications [100].

In a recent study, CRISPR-Cas9 system was applied in the AWRI2804 *Brettanomyces bruxellensis* strain [101]. This specie has been described as the principal spoilage yeast in the winemaking industry. From the enological point of view, *B. bruxellensis* is known for its high resistance to ethanol and ability to survive in low-nutrient, low-pH conditions, allowing for long-term proliferation in winemaking processes [102]. Using CRISPR-Cas9 in combination with gene transformation cassettes tailored for *B. bruxellensis*, the authors were able to delete *SSU1* genes (conferring sulfite tolerance) and provide the means for targeted gene deletion in this species.

5. Conclusion

Coupling traditional molecular genetic techniques with, synthetic biology and genome edition based on CRISPR, can enable the rapid optimization of wine yeasts [70]. Even though the era of yeast synthetic biology began in *S. cerevisiae*, it is swiftly expanding to non-*Saccharomyces* yeasts. However, genetic engineering in these yeasts is more challenging and limited by a lack of sophisticated genome editing tools yet and an incomplete knowledge of their genomes, metabolism and cellular physiology [103].

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Conflict of interest

The authors declare no conflict of interest.

Author details

Cecilia Picazo, Víctor Garrigós, Emilia Matallana and Agustín Aranda*
Institute for Integrative Systems Biology, I2SysBio, University of Valencia-CSIC, Paterna, Spain

*Address all correspondence to: agustin.aranda@csic.es

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Chapter 10

Determining Glucose Isomerase Activity in Different Wine Environments to Prevent Sluggish or Stuck Fermentations by Using Glucose Isomerase

Nahide Seray Kahraman and Haluk Hamamci

Abstract

The objective of this study was to determine glucose isomerase activity in different prepared original or synthetic wine media to prevent sluggish or stuck fermentation, which may be caused by sugar uptake deficiency in yeast. The unfermented grape juice contains almost equal amounts of glucose and fructose. After fermentation, the residual sugar is mostly fructose, this is called glucose/fructose discrepancy (GFD) and is caused by the affinity decrease of hexose transporters towards fructose as ethanol accumulates. This results in stuck fermentation and is unwanted as the wine is sweet and risks microbial spoilage. Converting remaining fructose to glucose by glucose isomerase may be a solution so we tested the activity of this enzyme in synthetic and original wine media. Glucose formation, 0.5 % w/v, from 1% w/v fructose took place in synthetic wine medium containing 13 % v/v ethanol, 1% w/v glycerol and at pH 3.3. In original wine medium glucose formation did not take place except when wine was diluted at least five folds and at pH values equal or higher than 6 whether if tartaric acid was present or not. Since neither dilution, nor pH adjustment can be applicable, other ways to employ this enzyme should be tried.

Keywords: glucose/fructose discrepancy, stuck fermentation, glucose isomerase, fermentation, yeast, wine media

1. Introduction

Wine fermentation is a complicated biochemical process in which yeasts play an active role in the production of ethanol, CO_2, and other metabolites from glucose and fructose of grapes [1]. Wine fermentation spontaneously takes place by yeast strains that are present on the grape surface or winery equipment. By today's technology, to achieve complete fermentation, good oenological properties and high production yield commercially produced yeast strains, and mostly *Saccharomyces cerevisiae* are used for wine fermentation as starter microorganisms [2].

S. cerevisiae strains derived from industrial wine have hexose transporters (HXT 1–7) that are responsible for wine fermentation. It is mentioned that there is

no growth or fermentation when HXT 1–7 are deleted from the genes of this yeast [3]. Ethanol formation in the wine medium causes a change in the affinities of hexose transporters and the change in the affinities of hexose transporters causes stuck fermentation. In this study, stuck fermentation due to ethanol formation was discussed and the experiments were conducted to prevent stuck fermentation.

The hexose sugars, glucose and fructose, are the main reducing monosaccharides present in grapes or grape musts. The amounts of total sugars in grapes or grape musts change between 160 and 300 g/L that consist of almost equal amounts of glucose and fructose before fermentation [4]. During wine fermentation, yeasts, especially *S. cerevisiae*, coferment these monosaccharides and produce wine components [3–5]. Since yeasts have glucophilic character, which is the preference of fermenting glucose to fructose [5], the utilization rate of glucose is higher than that of fructose during fermentation [4]. The glucophilic character of yeasts may be due to transportation across the plasma membrane of yeast by hexose transporters or phosphorylation inside the cell of yeast by hexose kinases has different affinities through glucose and fructose [4]. These different utilization rates result in glucose/fructose discrepancy (GFD) and residual fructose amount higher than 2 g/L [6] when the fermentation process is completed [4]. Since the sweetness of fructose is approximately twice than that of glucose [7], it affects the final sweetness of wine and the wine fermentation results in higher sweetness, which is undesirable in the wine industry. Also, high residual fructose increases the risk of microbial spoilage [8–10] and decreases the ethanol yield in wine [4–6]. This has been informed that sluggish (incomplete) or stuck (depleted) fermentation in the literature [6].

Although the exact reason for stuck or sluggish fermentations has not been determined yet, there are more than 15 reported reasons such as nitrogen deficiency [3, 4, 6, 8, 9, 11], limitation or excess amount of oxygen [8, 9], too much clarification [8, 9], formation of by-products due to fermentation [9], high ethanol accumulation [4, 6, 8, 9, 12], vitamin and mineral deficiency [8, 9], toxic residues for yeasts from fermentation [5, 8, 9], deprivation of nutrients for yeasts [10], too high or low temperatures [10], environment with high acidity [10], the formation of inhibitors like phenols [10], change in the equation of ionic components [10], higher sulfite content [8], and so on.

There are some known reasons for stuck fermentation and also, there are some possible ways and improvement methods against stuck fermentation such as nitrogen supplementation, controlling the oxygen amount, controlling the temperature of the environment, selecting the yeast according to process, controlling nutrients for yeast growth, and so on. Although many techniques and improvements are developed, stuck fermentation is still a major problem for the wine industry since it causes product losses [9].

Usage of an enzyme in wine media for preventing or restarting the stuck fermentation was not studied before. Therefore, using glucose isomerase to prevent or restart the stuck fermentation was studied as a novel approach.

2. Materials and methods

In this part, the "synthetic media" term was defined for different experiments that were in other parts, too. This term means that media are prepared with different wine components such as ethanol, glycerol, tartaric acid, calcium, and so on. To mimic the wine environment and activate the enzyme, 0.06 g $MgSO_4.7H_2O$ was added into 100 mL synthetic media solutions [13]. The experiments were conducted with 1 g of immobilized glucose isomerase added to 100 mL of solutions.

2.1 The effect of substrate type and temperature on the activity of glucose isomerase

In these experiments, either glucose or fructose was used as substrates of reactions at an amount of 1% w/v. The flasks were incubated in shakers at 60 or 30°C at 150 rpm. The pH values of media were 5.8 and 5.4 for glucose and fructose as substrates, respectively.

2.2 The effect of ethanol on the activity of glucose isomerase

To see the effect of ethanol on the enzyme, synthetic wine media were prepared by adding 13% v/v ethanol. Fructose was added to the media at an amount of 1% w/v. The pH of the solution was 5.5. The prepared flasks were incubated in shakers at 150 rpm. For comparing wine and wine without ethanol, samples were prepared with 1% fructose w/v. The flasks were incubated at 60°C for approximately 42 hours. The pHs of samples were 3.6 and 2.8 for wine with alcohol and without alcohol, respectively.

2.3 The effect of low pH values on the activity of glucose isomerase in synthetic wine medium

Different pH values, which were 3.3, 3.6, and 4, were adjusted with 1:1 acetic acid in synthetic wine media containing 1% w/v fructose. The flasks were incubated at 60°C and 150 rpm for almost 150 hours.

2.4 The effect of glycerol in synthetic wine media on the activity of glucose isomerase

The synthetic wine media were prepared with 1% w/v fructose, 13% v/v ethanol, and different glycerol contents. The flasks were incubated at 60°C for 47 hours. In order to simulate the wine environment, the pH was adjusted to 3.3 with 1:1 acetic acid solution.

2.5 The effect of sulfite content on the activity of glucose isomerase

The synthetic wine medium contained 1% w/v fructose as a substrate with different sulfite amounts. The flasks were incubated at 60°C and 150 rpm for 14.5 hours. The pH values of solutions were adjusted to 3.5 with 1:1 acetic acid solution.

2.6 The effect of tannins in red wine on the activity of glucose isomerase

In this experiment, 1 g of fructose was added to a 100 mL wine medium. Samples were incubated in flasks at 60°C and 150 rpm for almost 70 hours. The pH values of solutions were 3.37, 3.05, and 3.5 for Doluca Mistik Red, Turkey, 2016 (alcohol: 14.0% v/v) (w1), Doluca Mistik White, Turkey, 2016 (alcohol: 13.5% v/v) (w2), and Frontera, Chile, 2015 (alcohol: 12.5% v/v) (w3), respectively.

2.7 The effect of fermentation components on the activity of glucose isomerase

To obtain synthetic wine media with grape juice, 5% w/v fructose, 13% v/v ethanol, and 0.8% v/v glycerol were mixed with grape juice. Two types of grape juices were used during the experiments. The industrial white and red grape juices

that were obtained from the market were Kavaklıdere brand. Also, the homemade grape juice was tested and used during the experiments. Samples were incubated in flasks at 60°C and 150 rpm. Since grape juice already contained Mg^{+2} [14], the enzyme did not require an additional activator. The pH of homemade grape juice was 3.65 and 3.88 for synthetic wine media with homemade grape juice.

2.8 The effect of calcium content on the activity of glucose isomerase

In this experiment, 1% w/v fructose was used as a substrate, and 13% v/v ethanol and 0.8% v/v glycerol were added to provide synthetic wine environment. The pH values of the media were adjusted to 3.6 with 1:1 acetic acid solution. Since 100 mL red wine contains 8 mg calcium, the same amount of calcium must be present in the synthetic wine medium to provide the same conditions. Therefore, 0.015 g $Ca(OH)_2$ was added to 100 mL synthetic wine medium. The flasks were incubated at 60°C and 150 rpm for almost 65 hours. Another experiment was conducted by considering the ion retention capacity of EDTA to hold the calcium in homemade red wine media with alcohol content of 14% v/v. Different concentrations of EDTA were added to red wine media containing 1% w/v fructose as a substrate. The flasks were incubated at 60°C and 150 rpm. The pH of solutions with 0.1, 0.2, 0.6, 1, and 2% w/v EDTA were 3.46, 3.43, 3.53, 3.53, and 3.6, respectively. For the cation exchanger experiment, to test the enzyme activity in red wine media containing little amounts of calcium, 1% w/v fructose was added to sample numbers 0, 4, 6, and 10. The numbers of samples were named from 0 to 10. The number of 0 was the original wine sample, and other numbers were wine samples that were eluted through the resin at a rate of 40 mL per sample. While sample 0 contained almost 13 ppm calcium in it, other samples contained much less amounts of calcium with respect to sample 0. The pH values of samples were 3.6, and they were incubated at 60°C and 150 rpm for almost 80 hours.

2.9 The activity of glucose isomerase in diluted wine media

In the first experiment, homemade red wines were used containing 1% w/v fructose (added after dilution) as a substrate. Wines were diluted with distilled water at six different concentrations: 90, 70, 50, 30, 20, and 5% v/v and also 100% v/v red wine as a control. The prepared solutions were incubated at 60°C and 150 rpm for 42 hours. The pH values of solutions were 3.15, 3.15, 3.19, 3.3, 3.53, 3.59, and 4.45 for wines at concentrations of 100, 90, 70, 50, 30, 20and 5% v/v, respectively. Also, different brands and types of wines; w1, w2, and w3; at different concentrations, 100, 10, and 5% v/v, were used in another experiment. The samples containing 1% w/v fructose as a substrate were incubated at 60°C and 150 rpm for 27 hours. The pH values of solutions were 3.5, 3.45, and 3.03 for 100% v/v w3, w1, and w2, respectively.

2.10 The effect of tartaric acid on the activity of glucose isomerase

Synthetic wine environments with 1% w/v fructose and 0.3% w/v tartaric acid were prepared at pH values of 3.55 and 6.33 adjusted with 5 M NaOH and 24% w/v KOH solutions, respectively. Samples were incubated at 60°C and 150 rpm. For experiments, samples numbered as 0, 2, 5, 8, 9, 10, and 12 were chosen with the addition of 1% w/v fructose. The pH values of solutions, 0, 2, 5, 8, 9, 10, and 12, after fructose addition were 3.36, 9.85, 6.7, 4.8, 4.08, 3.83, and 3.66, respectively. Samples from 0 to 13 were passed through anion exchanger resin. The first 9 samples passed through an anion exchanger had higher pH values than the original sample. In the original sample, number 0, the tartaric acid content was equal to 0.248% w/v. Other samples from 1 to 13 did not contain any tartaric acid. The samples were incubated at 60°C and 150 rpm.

2.11 The effect of pH on the activity of glucose isomerase in red wine medium

Homemade wines at different pH values; 4, 5, 6, 7, and 8, containing 1% w/v fructose as a substrate were tested at temperatures of 60 and 30°C for this experiment. The pH adjustments of samples were done with a 5 M NaOH solution. The samples were incubated at 150 rpm, 60 and 30°C, for 70 hours.

2.12 Immobilized glucose isomerase

The enzyme that was used in this study was immobilized glucose isomerase and supplied from Cargill, Bursa. It was produced by Novozymes and the group of it was Sweetzyme IT Extra. The color of the enzyme was brown, and it was in the granulated form. Its approximate density value was 0.50 g/mL. Its typical activity range was above 400 IGIU/G (immobilized glucose isomerase unit per gram). The enzyme was stored at 4°C to avoid contamination.

2.13 Atomic absorption spectroscopy

Atomic absorption spectroscopy is a common method to detect metals and metalloids in liquid samples. Free atoms of gas are generated in the atomizer and they can absorb the radiation at a given frequency. By atomic absorption, the absorption of ground-state atoms in the gaseous state can be measured. The atoms make transitions to higher energy levels by absorbing the UV or visible light. The concentrations of metals or metalloids are determined from the absorption amount. To measure calcium content of the wine samples with an analytical method, atomic absorption unit (Jarrell Ash) was used at Middle East Technical University, Chemical Engineering Department.

2.14 HPLC (high-performance liquid chromatography)

In this study, the samples were analyzed to determine glucose, fructose, ethanol, glycerol, tartaric acid, malic acid, and acetic acid concentrations by using HPLC in Middle East Technical University, Food Engineering Department, Biotechnology Laboratory (Agilent Technologies, USA). The column and detector type of this HPLC was Rezex™ RFQ-Fast Acid H+ (8%) LC Column, 100 × 7.8 mm (length and internal diameter, respectively), and refractive index detector, respectively. The temperature of the refractive index detector and fast acid column was set to 30 and 25°C, respectively. 0.05 M H_2SO_4 was used as an eluent. For every sample, 10 μL of analyte was injected automatically with a flow rate of 0.6 mL/min.

3. Results and discussion

3.1 The effect of substrate type and temperature on the isomerization reaction of glucose isomerase

The equilibrium reaction from fructose to glucose or vice versa took place regardless of substrate type and temperature. According to the results of the experiments, the isomerization reactions were equilibrated at a faster rate when starting with glucose compared with fructose, especially at 30°C. Another important point resulting from experiments, the reactions took place at faster rates when conducted at 60°C compared with 30°C. Also, an equilibrium point was reached after 5 hours at 60°C while after 9 hours at 30°C (data not shown). As a result of these

experiments, it can be understood that the enzyme is suitable for both substrate types at different temperatures.

3.2 The effect of ethanol on the isomerization reaction of glucose isomerase

According to the results, glucose formation and fructose depletion were shown at 60 and 30°C. Also, it was shown that glucose concentration was slightly higher than fructose concentration after 25 hours at 30°C. However, fructose concentration was slightly higher than glucose concentration after 25 hours at 60°C. As it was mentioned before, the reaction took place more slowly at 30°C compared with 60°C by looking at the data (data not shown). As a consequence of this experiment, it was interpreted that 13% v/v ethanol did not inhibit the activity of the enzyme regardless of different temperature values.

Since ethanol did not inhibit the isomerization reaction in synthetic environment, an experiment was conducted by vaporizing ethanol in red wine to show the effect of ethanol on red wine. The ethanol concentration was reduced approximately from 13 to 0.3% v/v by keeping wine at air temperature on the magnetic stirrer. At the end of 42 hours, glucose concentrations of four samples did not change (data not shown). This means that the enzyme did not isomerize fructose even if there was no ethanol in the wine.

3.3 The effect of low pH values on isomerization reaction in synthetic medium

As seen in **Figure 1**, it was noticed that fructose was converted to glucose regardless of the pH values. Also, it was clearly noticed that the reaction at pH 4 was the fastest one when compared to others, whereas the slowest reaction was at pH 3.3 by looking at glucose formation rate. Therefore, it was thought that the reason for the inhibition of enzyme in the wine medium was not because of the low pH values. The rate of reaction decreased proportionally with decreasing pH, the inhibition effect in the wine medium was not due to acidic medium, and the effect of ethanol with low pH values had to be tested.

Under the same conditions in **Figure 1**, the glucose was formed under the same pH values with 13% v/v ethanol in medium as seen in **Figure 1**, too. The ethanol

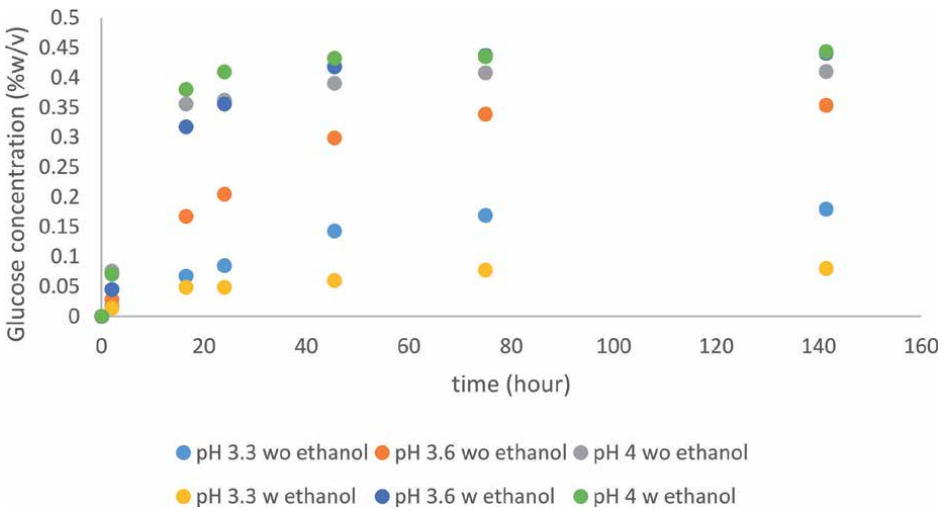

Figure 1.
Change of glucose concentrations at different pH values without and with ethanol.

effect experiments were conducted with the same amount of ethanol as experiments above, 13% v/v. The rate of reaction was obviously slower at pH 3.3. When without and with ethanol conditions were compared, almost the same amounts of glucose were formed for pH values of 3.6 and 4, whereas the amount of glucose at pH 3.3 was clearly lower in ethanol medium. Also, the glucose amounts formed at pH 3.6 and 4 were equalized at about the 45th hour with ethanol in the environment. The formed glucose amounts from 1% w/v fructose were nearly 0.2 and 0.1% w/v in medium without and with ethanol, respectively, at pH 3.3. As a consequence, glucose formation was detected in acidic media even in the presence of ethanol. The rate of reaction at pH 3.3 was clearly slower than others regardless of the presence of ethanol.

3.4 The effect of glycerol in synthetic media on the isomerization reaction

According to **Figure 2**, the isomerization reaction took place in all synthetic media with different glycerol concentrations. The rates of reactions were close to each other for all glycerol concentrations until the glycerol concentration was approached to 1% v/v. That is, the slowest reaction rate was obtained at the concentration of 1% of glycerol. It was easily concluded that the existence of glycerol in synthetic media containing ethanol did not inhibit the isomerization reaction.

3.5 The effect of sulfite content on isomerization reaction

Although it is known that glucose isomerase is used with sulfite [13], the experiments were also conducted in synthetic media with sulfite. After 14.5 hours of incubation, almost equal amounts of glucose were formed in all concentrations: 0.1, 0.04, and 0.01%, of sulfite (data not shown). Therefore, even higher sulfite contents compared to those present in wine did not inhibit the activity of glucose isomerase.

3.6 The effect of tannins in red wine on the isomerization reaction

It was thought that tannins might have an inhibitory effect on the isomerization reaction. Therefore, the comparison of glucose isomerase activity in red and white wine media was made. Glucose concentrations of all types of wines, w1, w2, and w3 remained stable during 70 hours of incubation. Normally, enzyme activity is seen in the first 2 or 3 hours of the experiments; in this case, no activity was observed even after 70 hours (data not shown). As a consequence, it was concluded that tannins had no inhibitory effect on the isomerization reaction since white wine also showed a similar behavior.

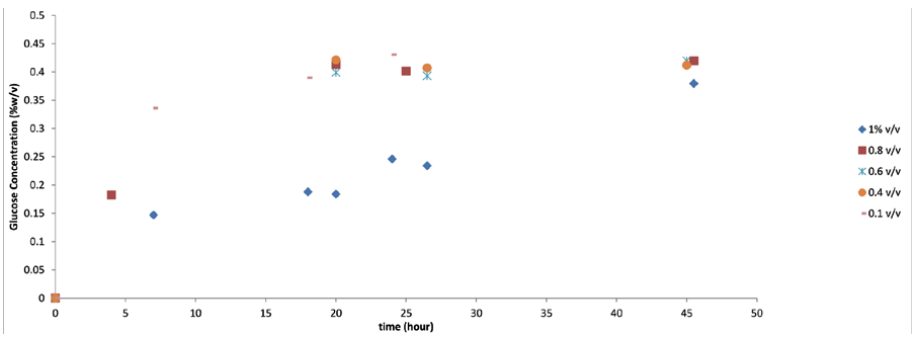

Figure 2.
Change of glucose concentrations in synthetic media with different glycerol contents during 47 hours.

3.7 The effect of fermentation components on isomerization reaction

Red grape juice contains almost equal amounts of glucose and fructose before fermentation. Therefore, to determine enzyme activity in grape juices, 5% w/v fructose was added into grape juice. As a result, glucose and fructose concentrations remained stable for 2.5 days. The fructose concentrations of samples were higher than those of glucose. Although samples were kept for 2.5 days, the enzyme did not isomerize the substrate in solutions (data not shown). As a result, since enzyme activity was not observed in grape must before fermentation, it was thought that a component that is present before fermentation would be inhibiting the enzyme.

3.8 The effect of calcium content on isomerization reaction

Generally, red wine contains both calcium and magnesium in amounts of 80 ppm and 120 ppm, respectively [15]. The ratio of magnesium to calcium must be equal to 12 to provide activating conditions for glucose isomerase. It was thought that the inhibition in the wine medium was because of the calcium content. In light of this information, experiments were conducted in synthetic media containing ethanol, glycerol and calcium, and red wine with the increased magnesium content. The conversion reaction took place in a synthetic medium without calcium; however, there was no formation of glucose in the synthetic medium with calcium (data not shown). As a result of this experiment, it was thought that calcium in wine may be inhibiting the glucose isomerase.

After the addition of 840 ppm magnesium into red wine, there was no formation of glucose in the red wine medium even with increased magnesium content at pH 3.28. The glucose formation was observed in red wine media with increased pH with or without additional magnesium. However, the reaction rate was faster at pH 7.5 than at pH 8.0 since the medium at pH 7.5 contained additional magnesium. The reactions reached equilibrium after about 50 hours (data not shown). As a result, it was concluded that the additional magnesium had no effect on low pH wine medium; however, it speeded the reaction rate at high pH wine media. Therefore, the effect of pH on isomerization reaction in wine media must be considered.

Another experiment was conducted by considering the ion retention capacity of EDTA to hold the calcium in homemade red wine media. There was no formation of glucose after 70 hours (data not shown). Therefore, it was concluded that EDTA had no positive effect on isomerization reaction in red wine media.

To test the enzyme activity in red wine media containing little amounts of calcium, samples 0, 4, 6, and 10 were chosen. The glucose concentrations of samples 0, 4, 6, and 10 remained stable and isomerization reaction did not take place after 80 hours (data not shown). Whereas calcium in synthetic medium inhibited the activity of glucose isomerase, there was also an inhibition effect in red wine even if there was no calcium in the environment.

3.9 The activity of glucose isomerase in dilute wine media

In the first experiment, homemade red wines were diluted with distilled water at six different concentrations. As seen in **Table 1**, except for concentrations at 20 and 5% v/v, the glucose concentrations of samples remained stable. At concentrations of 5 and 20% v/v red wines, the glucose formation took place during incubation.

Also, different brands and types of wines at different dilution concentrations were used in another experiment. As seen in **Table 1**, the glucose formation took place at concentrations of 10 and 5% v/v regardless of the brand type. Also, almost

Time	100% hand made	90% hand made	70% hand made	50% hand made	30% hand made	20% hand made	5% hand made	100% w1	10% w1	5% w1	100% w2	10% w2	5% w2	100% w3	10% w3	5% w3
0	0.274	0.247	0.192	0.137	0.078	0.05	0	0.098	0	0	0.068	0	0	0.274	0.025	0.012
6							0.156									
16	0.272	0.245	0.19													
16.5				0.151	0.108	0.242										
17								0.104	0.474	0.47	0.073	0.505	0.462	0.281	0.482	0.471
22	0.273	0.246	0.191													
24							0.463									
26								0.109	0.474	0.477	0.073	0.489	0.45	0.279	0.487	0.459
41.5				0.149	0.116	0.38										

Table 1.
Glucose concentrations of different wines with different dilution rates.

equal amounts of glucose formed at the same hours for different dilution factors and brand types.

As a result of these experiments, it was thought that a component or some components in wine coming from grapes may inhibit the isomerization reaction but this component or components may lose its or their effects in diluted wines but only at the levels of fivefold dilutions.

3.10 The effect of tartaric acid on the enzyme activity

The experiments were conducted with synthetic media containing fructose and tartaric acid and red wine containing no tartaric acid.

Fructose concentrations remained stable at 1% w/v at pH 3.55 and also, no glucose formation took place in flasks after 150 hours. However, glucose formation and fructose consumption were observed at pH 6.33. The reaction reached equilibrium within 30 hours with higher fructose contents (data not shown). As a result, L- (+)-tartaric acid inhibited the activity of glucose isomerase at pH 3.55; however, if the pH of the solution was 6.33, there was no inhibition with tartaric acid. Therefore, it was thought that if tartaric acid in wine is eliminated, the reaction would take place.

For experiments, samples numbered as 0, 2, 5, 8, 9, 10, and 12 were chosen. As seen in **Figure 3**, the 100% glucose formation took place at pH values of 9.85 and 6.7 and 50% glucose formation took place at 4.8, that is, sample numbers of 2, 5, and 8, respectively. Even if all samples did not contain tartaric acid, the conversion reaction took place at only higher pH values. Therefore, it was thought that the tartaric acid had an inhibitory effect at low pH values, under a pH of approximately 5. As a result, it had to be examined that glucose isomerase could or not convert fructose to glucose in wine media containing tartaric acid at high pH.

3.11 The effect of pH on isomerization reactions in red wine medium

As seen in **Figure 4**, the glucose formation took place at pH values of 6, 7, and 8 regardless of temperature. If temperature values were compared, the glucose concentration increased from 0.2% to almost 0.9% w/v for 60°C; however, it was from 0.2% to almost 0.4% w/v for 30°C during 70 hours of incubation. If pH values were compared, the reaction rates were higher for pH 8, 7, and 6 in decreasing order at 60°C. However, the reaction rate was almost equal for pH 7 and 8 but lower for pH 6 at 30°C.

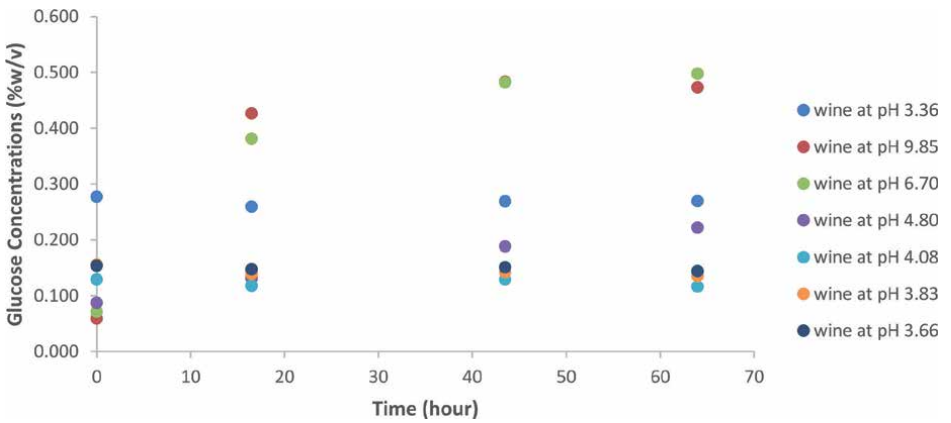

Figure 3.
Glucose concentrations of wine samples without tartaric acid.

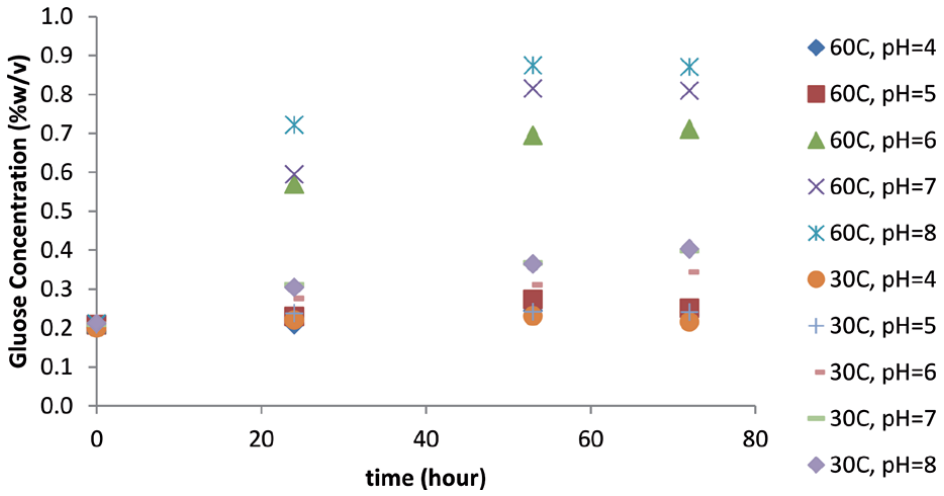

Figure 4.
Glucose concentrations of homemade red wines at different pH values at 60 and 30°C.

From the results of these experiments, the isomerization reactions took place in red wine media at pH values higher than 5 regardless of temperature. Therefore, it was thought that the pH of the medium had to be suitable in order for the glucose isomerase to be active in wine media.

4. Conclusion

In this study, the effects of different environmental and chemical factors on the activity of the enzyme glucose isomerase were tested with the final aim of using this enzyme for the conversion of fructose to glucose present in stuck wine fermentations.

To conclude, 0.5% w/v glucose formation from 1% w/v fructose took place in synthetic medium containing 13% v/v ethanol and 1% v/v glycerol at pH 3.3 and at temperatures of 60 or 30°C in approximately 48 hours. However, the glucose formation did not take place in synthetic medium if there was 0.3% w/v tartaric acid at pH 3.55, whereas glucose was formed at pH 6.33. In the original wine medium with dilution effect and at pH values equal or higher than 6, glucose was formed from fructose whether there was tartaric acid or not. Since dilution and increasing the pH of wine cannot be applicable, other ways to employ this enzyme to prevent stuck fermentation should be tried.

In final words, we can mention some methods that can be employed for stuck fermentations. A membrane system can be used for separating acetic acid from the wine medium [16]. This may increase glucose formation from fructose by using glucose isomerase. If a low pH resistant glucose isomerase can be searched and found, thanks to this enzyme, fructose be converted to glucose and stuck fermentation may be prevented. Finally, though not related to glucose isomerase, different yeast strains may be employed. The yeast strains usually employed in enology have glucophilic characters that prefer glucose against fructose. Fructophilic yeast strains, even if not employed at the start of the fermentations, may be added to stuck fermentations and may help consume the residual fructose in the medium.

Author details

Nahide Seray Kahraman[1] and Haluk Hamamci[1,2]*

1 Department of Food Engineering, Middle East Technical University, Ankara, Turkey

2 Department of Gastronomy and Culinary Arts, Alanya Hamdullah Emin Pasa University, Antalya, Turkey

*Address all correspondence to: hhamamci@metu.edu.tr

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Chapter 11

pH Control and Aroma Improvement Using the Non-*Saccharomyces Lachancea thermotolerans* and *Hanseniaspora* spp. Yeasts to Improve Wine Freshness in Warm Areas

Antonio Morata, Carlos Escott, Iris Loira, Juan Manuel Del Fresno, Cristian Vaquero, María Antonia Bañuelos, Felipe Palomero, Carmen López and Carmen González

Abstract

Lachancea thermotolerans is a yeast species that works as a powerful bio tool capable of metabolizing grape sugars into lactic acid via lactate dehydrogenase enzymes. The enological impact is an increase in total acidity and a decrease in pH levels (sometimes >0.5 pH units) with a concomitant slight reduction in alcohol (0.2–0.4% vol.), which helps balance freshness in wines from warm areas. In addition, higher levels of molecular SO_2 are favored, which helps to decrease SO_2 total content and achieve better antioxidant and antimicrobial performance. The simultaneous use with some apiculate yeast species of the genus *Hanseniaspora* helps to improve the aromatic profile through the production of acetyl esters and, in some cases, terpenes, which makes the wine aroma more complex, enhancing floral and fruity scents and making more complex and fresh wines. Furthermore, many species of *Hanseniaspora* increase the structure of wines, thus improving their body and palatability. Ternary fermentations with *Lachancea thermotolerans* and *Hanseniaspora* spp. sequentially followed by *Saccharomyces cerevisiae* are a useful bio tool for producing fresher wines from neutral varieties in warm areas.

Keywords: warm areas, wine, freshness, pH control, aroma, lactic acid, 2-phenylethyl acetate, non-*Saccharomyces*, *Lachancea thermotolerans*, *Hanseniaspora* spp.

1. Introduction

Global warming is leading to increased average temperatures and irrigation difficulties in some places due to water availability affecting vineyard and wine

production [1]. Wine regions affected by global warming have typical problems such as grape varieties with low acidity at harvest time, and high sugar contents that produce wines with flat taste, weak and simple aroma profile, and high alcoholic strength and pH [2]. Moreover, in red wines, the polyphenol content and especially the anthocyanins synthesis is affected, producing wines with less and more unstable colors [3]. Higher pHs make the wines less stable from a physicochemical point of view, but also more susceptible to microbial spoilage. In addition, higher pHs require strong acidity corrections, but pH is not easy to modify with tartaric acid, and wines are usually maintained at inadequate pH values. These values reduce the effectiveness of SO_2 by decreasing the molecular content that is more active as antimicrobial and antioxidant. The molecular SO_2 level of 0.6 mg/L has been proposed for maximum wine protection [4].

2. *Lachancea thermotolerans* and *Hanseniaspora* spp.

Yeast selection is a powerful tool to search for new strains with improved features that can enhance the sensory profile of wine or facilitate the technological process. Historically, vinifications have been performed with *Saccharomyces cerevisiae*, however, current enology is strongly focused on non-*Saccharomyces yeasts* [5]. Species such as: *Metschnikowia pulcherrima* [6], *Brettanomyces bruxellensis* [7], *Torulaspora delbrueckii* [8], *Aureobasidium pullulans* [9], *Hanseniaspora/Kloeckera* spp. [10], *Candida stellata* [11], *Saccharomycodes ludwigii* [12], *Starmerella bacillaris* [13], *Schizosaccharomyces pombe* [14], *Zygosaccharomyces rouxii* [15], *Wickerhamomyces anomalus* [16], *Lachancea thermotolerans* [17]. Most of them were used for their positive impact on wine aroma, flavor, mouthfeel, or color, and some of them were studied for their spoilage activity that may negatively affect wine quality.

This chapter is focused on the species *Lachancea thermotolerans* (Lt) (**Figure 1**) and the genus *Hanseniaspora* (H) spp. (**Figure 2**) because of their interesting behavior to improve the sensory profile and enhance the freshness of wines from warm areas. The main feature of Lt is the effective acidification by the formation of lactic acid from sugars [17]. Several lactate dehydrogenase sequences have been observed in the genome of Lt. Its morphology is similar to that of *Saccharomyces cerevisiae* (Sc) with ellipsoidal geometry and multipolar budding (**Figure 1**), although Lt shows a slightly smaller size. The use of Lt for wine acidification, pH control, and freshness improvement has been described

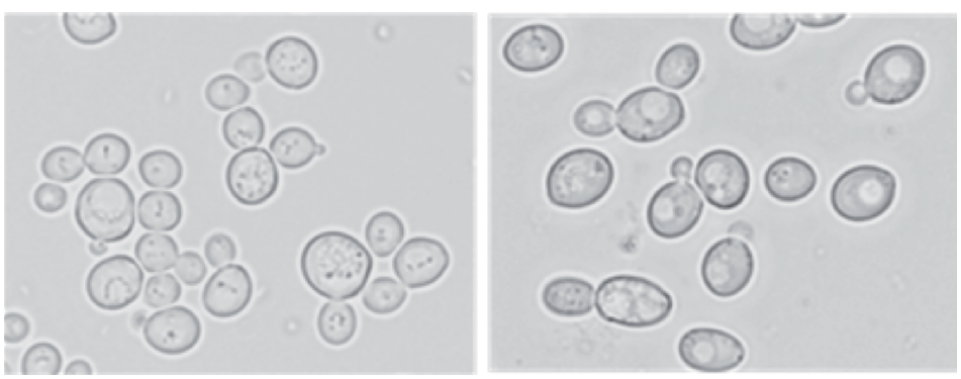

Figure 1.
Optical microscopy of Lachancea thermotolerans *(left) compared with* Saccharomyces cerevisiae *(right) both at different growth stages. Both species show an ellipsoidal shape with multipolar budding.*

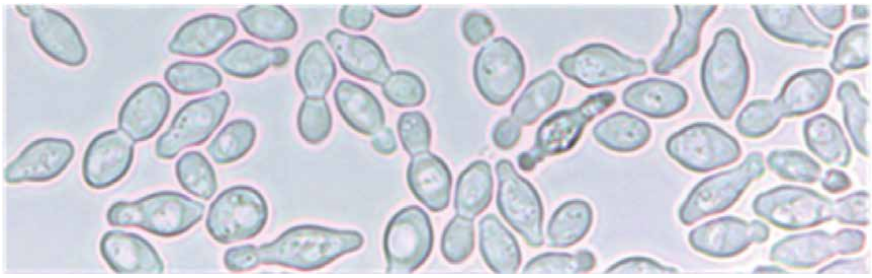

Figure 2.
Optical microscopy of Hanseniaspora vineae*, apiculate yeast with polar budding. Cells are in different stages of growth.*

in several works [18–24]. Acidification and pH control in warm areas is critical for wine quality and stability. A low pH not only produces fresher wines with a better sensory profile and improved consumer perception but also increases wine stability at the chemical and microbiological levels. So, wines with low pH are safer and more stable, and, as mentioned before, pH also favors higher molecular SO_2 content with higher antimicrobial and antioxidant performance. Therefore, biological acidification is a way to protect the wine and allows the reduction of SO_2 levels. The effect on molecular SO_2 at low pH has an impact on reducing the levels of spoilage microorganisms and, as a consequence, lowering the production of off-flavors and toxic molecules such as biogenic amines and others, thus producing safer and cleaner wines [25].

Lt shows a medium fermentative power with some strains reaching 9–10% vol. in ethanol [17]. In addition, Lt has shown other interesting features such as moderate volatile acidity [18, 22], even when used simultaneously with other species (*Metschnikowia pulcherrima*, *Hanseniaspora vineae*, *Torulaspora delbrueckii*) [23], and also reduction of volatile acidity levels in some conditions [26]. Furthermore, the positive role in the formation of thiol compounds in Sauvignon blanc has been described, releasing higher values of 3-Mercapto-1-hexanol (3MH) than the control yeast *Saccharomyces cerevisiae* (Sc) and significant contents of 4-Mercapto-4-methyl-2-pentanone (4MMP) compared to other non-*Saccharomyces* although, in this case, lower than Sc [27]. These thiol compounds are responsible for box tree (4MMP) and tropical fruit aroma (3MH) in wines that increase their complexity [28, 29]. Lt is a low producer of medium-chain fatty acids and their esters, therefore avoid heavy smells and flatness, which helps improve freshness [24].

The low pH produced by the intense biological acidification of Lt also has a positive effect on the color of white wine showing a bright and clean appearance and delaying the browning processes. This effect on browning is also evidenced by the higher levels of molecular SO_2 obtained at low pH which produces an intense antioxidant effect. Concerning red wine color, this reduction in pH favors an increase in color intensity by hyperchromic effect, but it also favors the stability of anthocyanins [30, 31].

In addition, we have observed that some Lt strains have an impact on wine structure, producing softer and full-bodied wines. However, this is not a typical feature of the Lt species, but only of some specific strains. It can be interesting to select these strains to achieve a good balance between acidity and mouthfeel.

Hanseniaspora species (*vineae*, *opuntiae*, *uvarum*, *guilliermondii*, *osmophila*, *valbyensis,* and others) are lemon-shaped apiculate yeasts with polar budding (**Figure 2**) that are typically found in grape juices at the onset of alcoholic fermentation [10], being included in the predominant indigenous yeast population of grapes. Most of them have a low fermentative power around or below 4% vol. However, some of them such as *H. vineae* can reach around 10% vol. [10].

Normally, *Hanseniaspora* spp. have been described as high producers of volatile acidity and have been removed from wine fermentation using SO_2 because of their high sensitivity to this antimicrobial agent. However, acetic acid production is quite variable among strains and some of them can reach values similar to those of Sc [32]. Some species such as *H. vineae* or *H. opuntiae* also show low values (<0.4 g/L) that can be comparable or lower than Sc [33, 34].

Several enzymatic activities have been described in *Hanseniaspora* spp., being especially interesting concerning aroma the expression of the β-D-glucosidase activity to release the free terpenes from their conjugated glucosides [35]. The latter compounds are found in higher concentrations in terpene-rich varieties, but due to their low volatility, they are odorless compounds. The use of non-*Saccharomyces* species with β-D-glucosidase activity is a way to increase wine aroma by releasing free terpenols.

Hanseniaspora vineae (Hv, anamorph sp. *Kloeckera africana*) [36] is one of the most interesting and trending species in enology, due to its medium-high fermentative power (up to 10% vol), its low volatile acidity, but especially for its high impact on wine aroma and structure. Some extra nutritional requirements have been described especially in thiamine, pantothenic acid, and YAN (yeast assimilable nitrogen) supplementation to avoid stuck or sluggish fermentations [10, 37]. The molecular proximity of Hv to Sc in phylogenetic trees is higher than that of other *Hanseniaspora* spp. (*H. opuntiae*, *H. guilliermondii*, *H., uvarum*) (**Figure 3**).

In addition to its interesting fermentative behavior with good implantation and suitable fermentation yield, Hv is useful to modulate the sensory profile of wines. The impact on the aroma is quite significant due to the formation of benzenoid compounds *de novo* by the chorismate-prephenate metabolic pathway (**Figure 4**). This pathway uses sugars as precursors and leads to the formation of floral benzenoid acetic esters such as benzyl acetate and 2-phenylethyl acetate [10, 36, 38, 39]. The production of 2-phenylethyl acetate among other fermentative compounds can

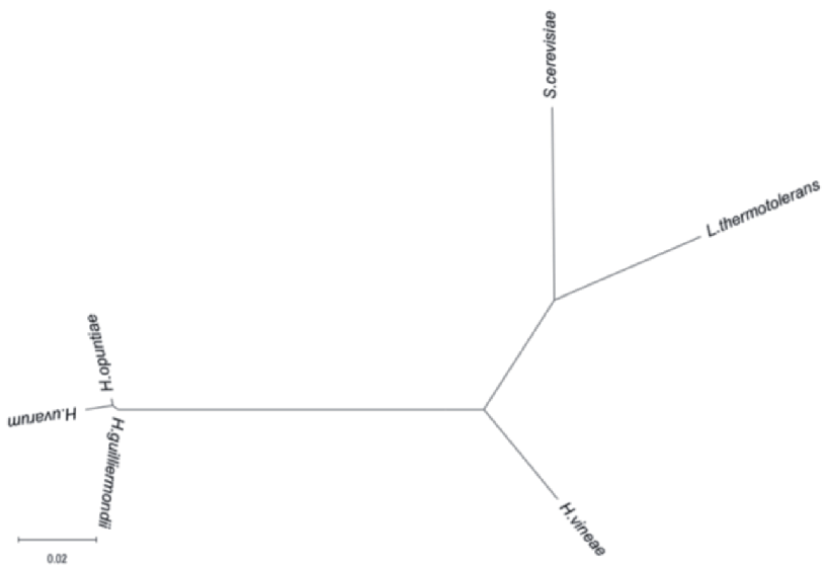

Figure 3.
Phylogenetic relationships among wine yeast species based on analysis of D1/D2 LSU rRNA gene sequences. The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model in MEGA7. GenBank access numbers follow strain numbers: Saccharomyces cerevisiae *NRRL Y12632/AY048154;* Lachancea thermotolerans *CBS 2803/KY108273;* Hanseniaspora uvarum *NRRL Y-1614/U84229;* Hanseniaspora opuntiae *CBS 8733/AJ512453;* Hanseniaspora vineae *NRRL Y-17529/U84224;* Hanseniaspora guilliermondii *NRRL Y1625/U84230.*

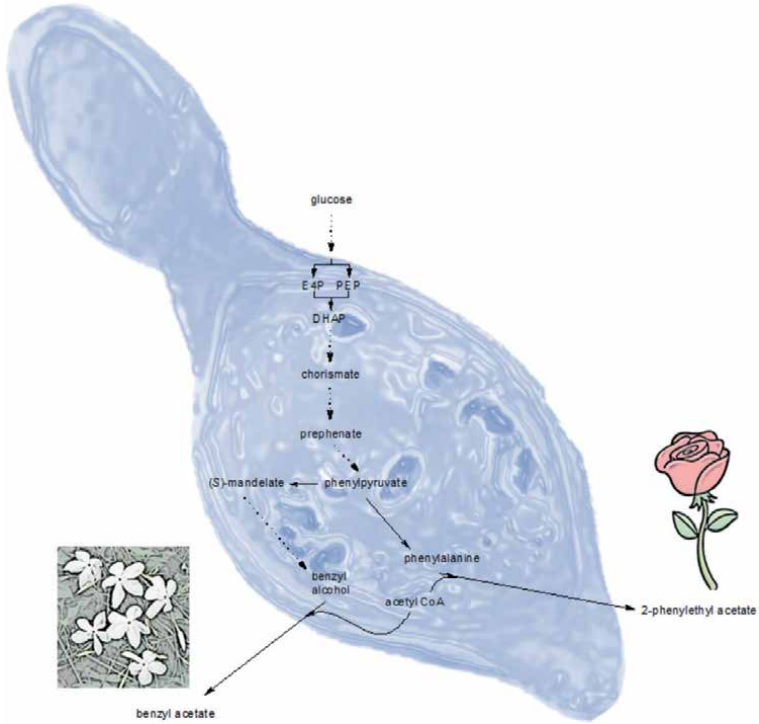

Figure 4.
De novo formation of floral esters by Hanseniaspora *spp. from sugars via the chorismate-prephenate-mandelate pathway. 2-phenylethyl acetate with rose petal aroma descriptor and benzyl acetate with jasmine aroma descriptor.*

separate, by PCA statistical analysis, the aromatic profile of Hv from Sc [34]. Benzyl alcohol concentrations in the fermentation of 11 Hv strains can reach x20-x200 the typical concentrations produced by Sc [38]. Benzyl acetate is the impact aroma of jasmine flowers and produces floral scents that help improve the sensory profile of wines produced from neutral grape varieties. Another impact compound in terms of floral aroma is 2-phenylethyl acetate, also produced by Hv. Its descriptor is rose petals and produces fresh floral perception in wines increasing complexity. This compound is also produced by other *Hanseniaspora* spp. such as *H. guilliermondii* [40], *H. uvarum* [41], *H. opuntiae* [42].

The impact of Hv on wine aroma is also related to the release or *de novo* formation of terpenes. Terpenes are aromatic compounds with a fruity and floral profile that enhance the aroma complexity and freshness of wines. Some grape varieties (Muscat, Gewürztraminer, Albariño) have terpenes produced by the plant in the form of terpenes bonded to sugars as a way to better translocate the hydrophobic free terpenes through the plant tissues. Bonded terpenes are more polar but less volatile, so less aromatic. Hv can express extracellular β-D-glucosidase releasing free terpenes during fermentation and thus improving the varietal aroma of wines [10, 35, 43]. The β-xylosidase activity has also been described in Hv [43].

De novo formation of terpenes from sugars has also been observed in fermentations with Hv. In the fermentation of the neutral variety Macabeo, the formation of a significant concentration of α-terpineol (>100 μg/L) has been observed, but below its sensory threshold [36]. Sequential fermentations with Hv followed by Sc in Albillo grapes have shown much higher concentrations of terpenes (316 μg/L) than with Sc controls (114 μg/L) [44]. Linalool, β-citronellol, and geraniol showed

higher concentrations than in the Sc control (>x3, >x4, and > x2 respectively), but also above their respective sensory thresholds [44]. The balsamic terpenes terpinene-4-ol and α-terpineol were also at significantly higher concentrations but below the sensory threshold. Furthermore, several polyoxygenated terpenes showed significantly higher concentrations, but they usually have higher sensory thresholds and, therefore, less impact on the aroma.

Another interesting impact of some *Hanseniaspora* species is the effect on wine structure. Usually, wines fermented by these yeasts show a full-bodied structure and better palatability in the mouth. Fermentation of Macabeo grape must with Hv has shown a sensory profile where tasters perceived improved structure and volume [10]. When the contents of cell wall polysaccharides released by Hv were measured by size exclusion chromatography no significant differences were found with Sc. However, the absorbance at 280 nm, which can be correlated with protein, shows higher values especially at the end of fermentation with Hv [34]. When aging on lees (AOL) is extended for several months, there are no differences between Hv and Sc control. The use of size exclusion chromatography showed slightly higher molecular sizes in the polysaccharides released by Hv that may influence the more intense mouthfeel [44].

3. Use of *Lachancea thermotolerans* and *Hanseniaspora* spp. at industrial scale

The use of a new non-*Saccharomyces* strain requires a lot of experimental research in the laboratory, but also several years of pilot, semi-industrial and industrial-scale trials. **Table 1** details the fermentations, years, wineries, regions, varieties, volumes, controls, and pH effects of selected *Lachancea thermotolerans* strains L31 and A54, currently under industrial evaluation by Lallemand. The strains were tested on white and red grape varieties to see the implantation and performance of acidification on settled white must, but also on crushed red grapes with skins and seeds. Volumes ranged from 500 to 12,000 in white musts and from 1,000 kg to 15,000 kg in crushed red grapes.

In all conditions, acidification was quite effective, even in crushed grapes where the high presence of indigenous yeasts can affect the implantation by reducing the prevalence of the Lt strain. It is interesting to highlight that acidification is effective in varieties with low pHs such as Albariño (3.1) and varieties with high initial pH

Variety	Region	Scale	Year	Strain	Effect on pH	Lactic acid (g/L)
Albariño (white)	Rias Baixas	500 L	2016	L31	3.12 → 2.85	2.7
Tempranillo (red)	Ribera del Duero	1,000 kg	2017	L31	4.20 → 3.63	6.6
Tempranillo (red)	Ribera del Duero	15,000 kg	2020	L31	3.8 → 3.66	2.3
Tempranillo (red)	Mancha	8,000 kg	2020	L31	3.84 → 3.34	9.4
Airén (white)	Mancha	12,500 L	2020	A54	3.75 → 3.47	2.0

Table 1.
Performance of Lachancea thermotolerans *L31 & A54 strains on several semi-industrial trials.*

such as Airén or Tempranillo (3.75–4.20). In terms of potential alcohol, the varieties showed alcoholic strengths ranging from 11 to 12% vol. in the whites and 14–15% in the reds.

Volatile acidity was quite moderate and ranged from 0.38 to 0.46 g/L. The other fermentative volatiles were at normal values for the wines, only the ethyl lactate content was higher than the Sc controls (40–50 mg/L) due to intense lactic acid production, but below the sensory threshold for this ester (150 mg/L) [22].

It is important to note that when Lt strains are used on an industrial scale on real musts or crushed grapes it is important to keep the total SO_2 concentration below 20 mg/L. Otherwise, Lt implantation and development can be seriously affected. The typical acidification pattern shows maximum lactic acid production at the beginning of fermentation (days 3–6, **Figure 5**) depending on inoculation rate, temperature, nutrients, and must composition [22, 23, 45].

It can be observed how the high pH typical of varieties such as Tempranillo in warm areas is deleterious to wine quality, not only producing chemical and microbial instability but also making sulfites inefficient due to low molecular SO_2 levels. The natural biological acidification of Lt produces pH reductions from 4.0 to 3.5 or less resulting in molecular SO_2 levels increasing from <0.4 (dangerous) to >0.8 (safe) [25]. It should also be noted that lactic acid is a stable acid that cannot be altered or metabolized by microorganisms during wine aging. In addition, at high doses (>4 g/L) it inhibits malolactic fermentation, which can be interesting to maintain extra acidity and protect the freshness in wines from warm areas [46].

From a sensory point of view, biological acidification produces a citric freshness, which can be very crispy at high concentrations but can never be perceived as dairy acidity. This is because the milky profile of malolactic fermentation and fermented milk comes from some secondary metabolites such as acetoin or diacetyl that are found in low concentrations in Lt fermentations.

The typical sensory profile of Lt normally shows increased freshness with improved acidity (**Figure 6**) which, depending on the level of acidification, can be somewhat unbalanced and crispy. This can be controlled by the timing of Sc inoculation in sequential fermentation or, subsequently, by blending Lt wines with Sc wines. Even when Lt does not have a strong impact on the aroma, the profile is fresh, fruity, and pleasant. The body in the wines is similar to that of Sc, but, as noted above, specific strains have effects on palatability.

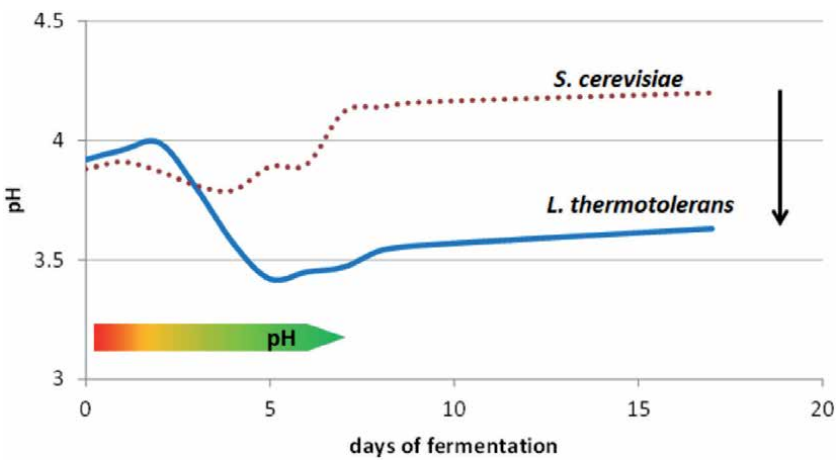

Figure 5.
Typical pH evolution in industrial fermentations driven by Lachancea thermotolerans*. The gradient color scale shows the safety of wines in terms of microbial and chemical stability as a function of pH.*

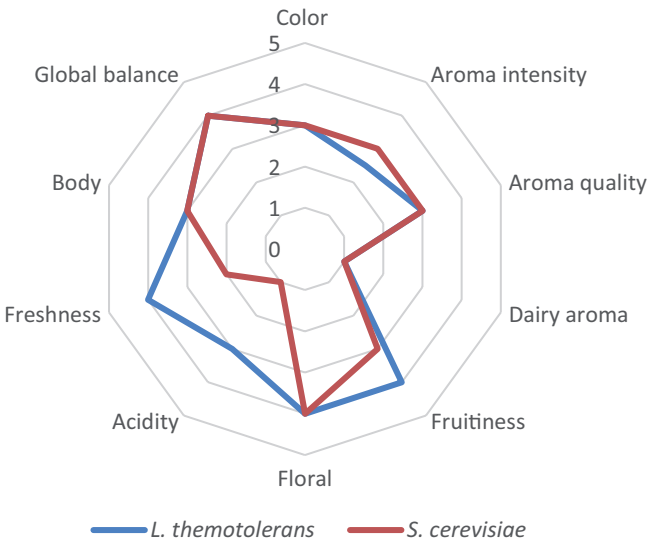

Figure 6.
Comparative sensory spider net of fermentations with Lachancea thermotolerans *and* Saccharomyces cerevisiae.

Additionally, we have compared in Airen fermentations the effect of 72 h of biological acidification with Lt (2 strains: L31 and Laktia from Lallemand) with chemical acidification using 1.5 g/L tartaric acid. Natural biological acidification produced the same effect on pH without using chemical additives [47]. Furthermore, chemical stability is higher due to the high potassium salts precipitation produced during chemical acidification with tartaric acid.

Concerning the use of *Hanseniaspora* spp. on an industrial scale, the most important species are *Hanseniaspora vineae* and *H. opuntiae*, although *H. uvarum* has also been used to some extent. We have experience fermenting Albillo (*Vitis vinifera* L.) white variety with *H. uvarum* in stainless steel and oak barrels to produce white wines aged on lees or blends of Albillo and Tempranillo (*Vitis vinifera* L.) to produce rosé wines (**Table 2**). Moreover, we have fermented must from Airen (*Vitis vinifera* L.),

Variety	Region	Scale	Year	Strain	Aroma	Mouthfeel/ Color
Albillo (white)	Ribera del Duero	150 L Stainless steel barrels	2019	Hv T02/5A	terpenes (x3) 2phenylethyl acetate (x1.33)	Improved palatability
Albillo and Tempranillo (rosé)	Ribera del Duero	150 L Stainless steel barrels	2020	Hv T02/5A	2phenylethyl acetate (x1.65)	Improved palatability Better color (red-bluish)
Albillo and Tempranillo (rosé)	Ribera del Duero	150 L Oak barrels	2020	Hv T02/5A	terpenes (x2.5)	Improved palatability Better color (red-bluish)
Airén (white)	Mancha	12,500 L	2020	Ho A56	2phenylethyl acetate	Improved palatability

Table 2.
Performance of Hanseniaspora *spp. on several semi-industrial trials.* Hanseniaspora vineae *(Hv),* Hanseniaspora opuntiae *(Ho).*

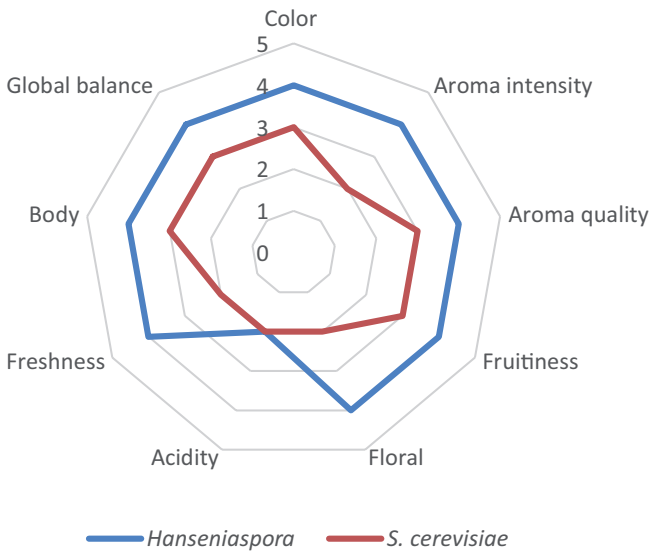

Figure 7.
Comparative sensory spider net of fermentations with Hanseniaspora vineae/opuntiae *and* Saccharomyces cerevisiae.

a neutral flat grape variety, in large stainless-steel tanks using *H. opuntiae*. This species enabled the production of wines with more body, better palatability, and floral aroma.

The formation of terpenes and floral esters by *Hanseniaspora* spp. has an interesting impact on the sensory profile, especially with neutral grape varieties such as Airén or Albillo that express fruitier and more floral wines with greater aromatic freshness. In addition, a positive effect on color can be found in rosé wines with higher anthocyanin contents in fermentations with Hv and especially some acylated derivatives [48]. **Figure 7** shows the typical sensory profile of *Hanseniaspora* spp. compared to *Saccharomyces cerevisiae*.

4. Biocompatibility

Lt and Hv/Ho can be used in mixed fermentations or independent fermentations, subsequently blending both wines in appropriate quantities. When used in mixed fermentations, biocompatibility must be taken into account due to the special sensitivity of *Hanseniaspora* to vitamins such as thiamine and pantothenate or nitrogen contents. Nutritional deficits can lead to the low formation of acetate esters and terpenes with the consequence of a low impact on the aroma. A similar situation is observed in *Lachancea thermotolerans* in which nutritional imbalances affect implantation and development of the yeast population and therefore low acidification compromising the effect on pH. Lower acidification has been observed in ternary fermentations with Lt and Hv sequentially followed by Sc under standard nutritional conditions [45]. The development of further research to carefully optimize the nutritional and physicochemical conditions (temperature, SO_2, pH) for interspecies compatibility will be a key parameter for the successful application of this biotechnology.

5. Conclusion

The combined use of *Hanseniaspora* spp. (*vineae* or *opuntiae*) with *Lachancea thermotolerans* in mixed fermentations subsequently finished sequentially by

Saccharomyces or the independent use of them and later blending their wines is interesting biotechnology to improve flat neutral varieties by increasing acidity, aroma, body, and color, and thus improving the sensory profile and freshness. Several considerations have been described to achieve successful fermentations in terms of nutritional aspects to develop and yeasts biocompatibility.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Antonio Morata*, Carlos Escott, Iris Loira, Juan Manuel Del Fresno, Cristian Vaquero, María Antonia Bañuelos, Felipe Palomero, Carmen López and Carmen González
EnotecUPM, Technical University of Madrid, Madrid, Spain

*Address all correspondence to: antonio.morata@upm.es

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Section 3

Enology and Stabilization

Chapter 12

Microbial Decontamination by Pulsed Electric Fields (PEF) in Winemaking

Carlota Delso, Alejandro Berzosa, Jorge Sanz, Ignacio Álvarez and Javier Raso

Abstract

Pulsed Electric Fields (PEF) is a non-thermal technique that causes electroporation of cell membranes by applying very short pulses (μs) of a high-intensity electric field (kV/cm). Irreversible electroporation leads to the formation of permanent conductive channels in the cytoplasmic membrane of cells, resulting in the loss of cell viability. This effect is achieved with low energy requirements and minimal deterioration of quality. This chapter reviews the studies hitherto conducted to evaluate the potential of PEF as a technology for microbial decontamination in the winemaking process for reducing or replacing the use of SO_2, for guaranteeing reproducible fermentations or for wine stabilization.

Keywords: PEF, SO_2, electroporation, microbial inactivation, wine, pulsed electric fields

1. Introduction

Winemaking is a complex process that extends from grape cultivation and harvesting to wine consumption. In the course of this process, many different chemical, physical, microbiological, and sensory reactions are involved. Microorganisms play an essential role, since alcoholic fermentation and frequently also malolactic fermentation are fundamental steps in winemaking. During these fermentation steps, the evolution of certain chemical compounds depends directly on their interaction with microorganisms, thereby resulting in many of the characteristic and desirable flavors in wine [1]. Conversely, microorganisms can also contaminate and spoil the wine in several steps of the winemaking procedure, causing re-fermentation, off-flavors, volatile acidity, and bottle explosion. Moreover, microorganisms can produce compounds that are hazardous for human health, such as biogenic amines [2]. The ultimate quality of wines and their commercial value are therefore directly associated with those microflora which are beneficial; nevertheless, microbial spoilage of wine can lead to a number of drawbacks and economic losses for the wine industry. It is thus essential to monitor the entire winemaking process in the endeavor to avoid contamination caused by microorganisms. This can be achieved using chemical preservatives and/or certain physical treatments designed to inactivate microorganisms, inhibit their growth, or directly separate them physically from wine.

The main yeasts regarded as true spoilage strains in wine are *Brettanomyces bruxellensis*, *Zygosaccharomyces bailii*, and *Saccharomyces cerevisiae*. *B. bruxellensis* is one of the most undesirable strains in wineries, as even at very low concentrations it can produce the typical "horse sweat" taint, and early detection is difficult [3]. Because of its tolerance to high sugar and sulfur dioxide concentrations, *Zygosaccharomyces* may cause turbidity, produce CO_2, and even re-ferment sweet wines and grape juices [4]. *S. cerevisiae*, although involved in the alcoholic fermentation process, can be responsible for wine spoilage when a nutritional imbalance in the grape juice triggers off-flavor production. Other species of the genera *Kloeckera/Hanseniaspora*, *Pichia*, and *Candida* can also produce film layers and undesired metabolites [5].

Lactic Acid Bacteria (LAB) are responsible for malolactic fermentation (MLF), but can also negatively affect the quality of wines as spoilage microorganisms when they proliferate at the incorrect time during winemaking [6]. Wine-associated microbial LAB genera are *Lactobacillus, Leuconostoc, Oenococcus*, and *Pediococcus*. LAB growth in wine can imply the production of undesirable aroma and flavor compounds, biogenic amines, acrolein, and ethyl carbamate, or can cause a slimy appearance. In the category of Acetic Acid Bacteria (AAB), the three main associated genera considered as spoilage bacteria in wines are *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter*. Their principal effect on wines is the production of acetic acid, acetaldehyde, and ethyl acetate, which confer sour, nutty, and solvent-like flavors, respectively. All these groups of spoilage microorganisms in wine have in common their ethanol tolerance, their ability to grow at low pH (< 4.0), and, in some cases, a high tolerance to SO_2. In order to establish a methodology for must or wine decontamination and stabilization, it would be necessary to establish which are the target microorganisms in the different steps of winemaking, and to study their tolerance/resistance to the chosen lethal agent.

2. Current innovative strategies for microbial decontamination in winemaking

At present, the main strategy applied to control spoilage microorganisms along the winemaking process is the addition of sulfur dioxide (SO_2), a compound which is able to ensure antioxidant protection and microbiological stability. Although SO_2 is a highly effective and inexpensive preservative widely used in the wine industry, concerns have been raised regarding its potentially adverse effects on human health. The general trend in the wine industry is thus currently to reduce SO_2 content, or even to eliminate it altogether [7].

Dimethyl dicarbonate (DMDC), lysozyme, and sorbic acid are chemical compounds proposed as alternatives to SO_2, and they are already allowed as antimicrobials in winemaking by the OIV. Although they have proven effective against certain wine spoilage microorganisms, at their maximum permitted doses none of them is sufficiently effective against the entire range of microorganisms of concern [7].

Microfiltration, on the other hand, is a common physical procedure applied in winemaking for purposes of microbial stabilization. However, this technique is only applied before bottling and has some drawbacks due to its potentially deleterious effects on flavor and color properties of wines, depending on filter media and intrinsic wine characteristics. Sterile filtration presents further practical problems associated with frequent fouling, the high cost of filters, their management, and the possible recontamination of wines during bottling [8]. Heat treatments, despite their well-known high efficacy in terms of microbial inactivation, are not commonly used in wineries due to the negative effects of high temperature on the valuable sensory properties of wine [9]. Generally, thermal pasteurization is only applied to low-medium quality wines prior to bottling.

Similarly, emerging preservation techniques have been proposed for the microbial stabilization of wines. High hydrostatic pressure (HHP) is one of the most widely studied methods, and it has proven effective against most of the target microorganisms in wine [10]. However, due to the necessity of treating bottled wine and the possible acceleration of unwanted chemical reactions, along with the high cost and small flexibility of HPP devices, is ultimately not the most feasible technique for wineries [11]. Ultrasound, ultraviolet light, ionizing radiation, ultra-high pressure homogenization (UHPH), and microwaves have been also investigated for wine, for must, and even for barrel sterilization [12–17]. The main recent studies have focused on these techniques' lethal efficacy, but it is still necessary to obtain further knowledge about their effects on sensory quality and their actual feasibility at an industrial scale. Moreover, none of these innovative physical technologies is yet approved for wine stabilization by the OIV, except for HHP and UHPH.

In order to meet consumer demands, the wine industry is thus attempting to find new strategies to reduce or eliminate the use of SO_2. However, the chosen alternative technique should ensure that the levels of inactivation required for stabilization are achieved in each step of the winemaking process, without any detectable effect on sensorial and physicochemical properties of wine.

3. Fundamentals of pulsed electric fields technology

During processing with pulsed electric fields (PEF), products are subjected to very short pulses (μs) of high voltage (kV). The applied external voltage generates an electric field which, if intense enough, causes an electrical breakdown of the cell's cytoplasmic membrane. This phenomenon, referred to as electroporation, may cause the inactivation of vegetative cells of microorganisms, among other effects. The capability of PEF to inactivate microorganisms at temperatures that do not affect the flavor, color, or nutrient value of foods is highly attractive for the food industry.

3.1 Principles of PEF processing

PEF processing involves the intermittent application of direct-current voltage pulses (kV) for very short periods through a material placed between two electrodes. A typical PEF setup for food processing therefore includes a charging unit, an energy storage unit, and a switching unit that triggers pulse formation and releases the electrical pulses in the treatment chamber (**Figure 1**) [18]. According to the triggering system used for discharging the stored energy, the shape of the pulses delivered in the treatment chamber is either exponential or square. A PEF treatment chamber is composed of two electrodes held in position by insulating material, which forms an enclosure to contain the product to be treated. Parallel electrode and collinear configuration are the two proposed designs for the microbial decontamination of liquid foods by PEF [19]. Parallel electrode configuration hinders the formation of a uniform electric field in the treatment zone, whereas in a collinear treatment chamber the distribution of the electric field in the treatment zone is inhomogeneous. Nevertheless, the collinear chamber's higher load resistance, the configuration's overall lower energy requirements, and the circular section similar to the pipes used in food processing plants are nevertheless the reasons why collinear chambers are the ones currently used in industrial applications.

The effectiveness of PEF processing depends on several parameters, among which the ones most often used to describe the intensity of an applied PEF treatment are: electric field strength, processing time, total specific energy input, and

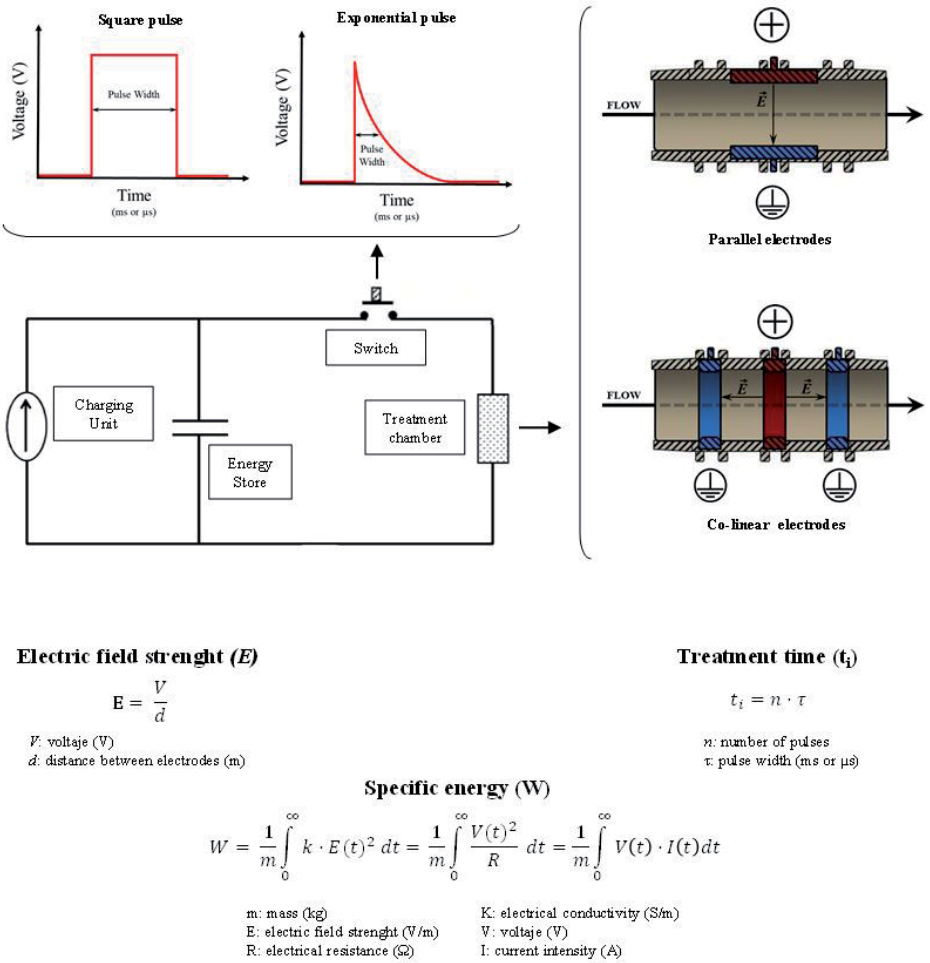

Figure 1.
Simplified diagram of an electrical circuit of a PEF generator. The different pulse shapes (exponential or square) and chamber geometries (parallel and collinear electrodes) used for the application of PEF treatments in continuous conditions are plotted. The main processing parameters of PEF technology are shown below.

temperature (**Figure 1**). Electric field strength depends on the external voltage applied, as well as on the distance between the electrodes. Treatment time represents the product's exposure time to the electric field, and depends on the number of applied pulses as well as on the pulse width. The treatment's specific energy (energy applied per mass unit) is dependent on the applied voltage, the pulse width, the number of pulses and the treatment chamber's resistance. Treatment chamber resistance varies according to its geometry and the product's conductivity. Finally, temperature is the other parameter to be considered in the evaluation of the efficiency of PEF processing in microbial inactivation. Inactivation usually increases at a higher temperature of the treatment medium – even within temperature ranges that are not otherwise lethal for microorganisms [20].

3.2 Effects of an external electric field on microorganisms

After the application of a PEF treatment, the presence of nucleic acid, proteins, and other components of the microbial cytoplasm such as adenosine triphosphate (ATP) has been observed in the medium surrounding the microorganisms. These

observations suggest that PEF causes the formation of local defects or pores (electroporation), thereby leading to an increment of cell membrane permeability. Depending on the intensity of the treatment applied (electric field strength, processing time, specific energy) and cell characteristics (size, shape, orientation within the electric field), the electroporation of the cytoplasmic membrane can be either reversible or irreversible. It is reversible if the bilayer returns spontaneously to its initial state by recovering membrane integrity. If structural changes in the lipid bilayer due to PEF treatment are permanent, electroporation is irreversible. Permanent electroporation causes uncontrolled molecular transport across the membrane, hinders the cells' homeostatic capacity, and eventually leads to microbial death.

The electroporation of the cytoplasmic membrane caused by PEF indicates that this technology could be an effective procedure the inactivation of vegetative bacteria cells. But bacterial spores, which are a resting stage of some bacteria such as *Bacillus* and *Clostridium*, are resistant to these treatments. The low water content and unique cellular structure of bacterial spores, consisting of several layers surrounding the core, seem to provide resistance to the effect of the external high-intensity electric field generated during PEF processing.

4. Application of PEF for microbial decontamination in wineries

PEF treatments have been shown to cause microbial inactivation of vegetative cells of bacteria, yeast, and molds. Bacterial spores are resistant to PEF; nevertheless, since spores are not able to proliferate under acidic conditions, PEF represents a worthwhile alternative for the stabilization of acidic food such as must and wine. To implement PEF technology as a preservation method in wineries, it would be essential to determine the target microorganisms in every step of its application, and to conduct studies to prove that it ensures the level of microbial decontamination required to avoid spoilage. Finally, optimized PEF conditions should be applicable at an industrial scale without any negative effect on the appreciated quality properties of wine.

Several studies have demonstrated the potential of PEF for the inactivation of bacteria and yeast in must and wine. **Figure 2** shows the different winemaking steps in which the effectiveness of PEF for microbial decontamination and/or control of the microbial population in must or wine has been investigated. The main results obtained in those studies are described below.

4.1 Application of PEF for decontamination of must

PEF has proven highly effective in the inactivation of diverse microorganisms present in several kinds of fruit juice, including grape juice [21–23]. Reduction rates ranging from 2.0 to 4.0 log cycles were obtained by PEF (35 kV/cm, 1 ms) in must contaminated by a mixture of spoilage yeast and bacteria, such as *Saccharomyces cerevisiae, Kloeckera apiculata*, *Lactobacillus plantarum*, *Lactobacillus hilgardii*, and *Gluconobacter oxydans* [24]. In that study, the lethality of PEF was higher for yeast than for bacteria. Wu et al. achieved 4.0 log cycles of reduction in the natural spoilage flora of grape juice by applying a more intense PEF treatment (80 kV/cm, 40 μs) at 50°C that did not affect the juice's vitamin C content [25]. Further inactivation rates (up to 5.0 log cycles) were obtained when PEF was combined with certain antimicrobials such as lysozyme and nisin. Puértolas et al. established an optimum treatment of 186 kJ/kg at 29 kV/cm, reducing 99.9% of the spoilage flora of artificially contaminated must [26]. Moreover, PEF treatments have been shown

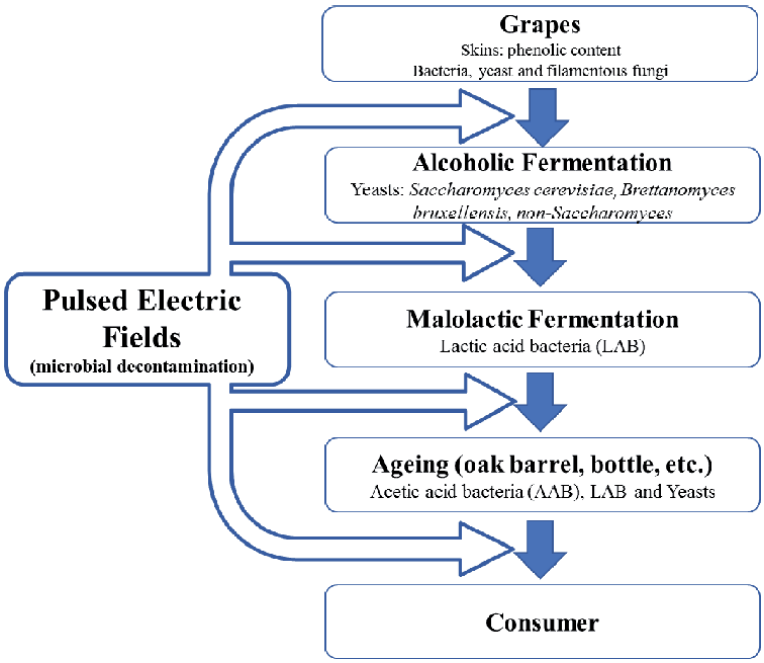

Figure 2.
Steps of winemaking in which pulsed electric fields have potential application for microbial control and decontamination.

to cause no significant changes in the physicochemical and nutritional properties of must, even when they are combined with mild temperatures (<50°C) [27, 28].

Studies in near-actual winemaking conditions have been conducted to evaluate the potential of PEF for replacing SO_2 prior to alcoholic fermentation, with the objective of stabilizing the must and thus facilitating the growth of the culture starters. PEF treatments in must at 35 kV/cm for 1 ms was shown to be effective for controlling the microbial population before the inoculation of the yeast strains selected for alcoholic fermentation. The wines obtained after the alcoholic fermentation of PEF-treated must do not show any change in terms of their volatile profile, nor any modification of their characteristics after subsequent aging in bottles in comparison to wines added with SO_2 [29, 30]. Alternatively, the use of non-*Saccharomyces* strains for alcoholic fermentation and for the improvement of the sensorial profile of neutral varieties is becoming a new trend in winemaking. Certain studies have confirmed that non-*Saccharomyces* yeasts implant themselves better in PEF-treated must [31, 32]. Consequently, higher levels of several specific metabolites of interest produced by non-*Saccharomyces* yeasts have been detected in wines obtained from PEF-treated musts.

Therefore, must stabilization by PEF is proving to be a good alternative for the reduction or elimination of the SO_2 dose, thereby facilitating the implementation of selected *Saccharomyces* and non-*Saccharomyces* yeast starters for purposes of alcoholic fermentation.

4.2 Application of PEF for wine decontamination after alcoholic fermentation

Although *S. cerevisiae* strains are predominant in wine after alcoholic fermentation (AF), certain other non-*Saccharomyces* yeasts may persist due to their ethanol tolerance. Not only yeasts, but also LAB and AAB from grapes and even other microbes present in winery facilities or in the environment can contaminate

the wine. Some wines are subjected to malolactic fermentation (MLF) after AF. Generally, starter cultures of LAB are added to the freshly fermented wine to ensure good implantation and prevent the proliferation of undesirable bacteria. The usual addition of SO_2 prior to MLF can limit or hamper the implantation of the selected starters. PEF has thus been studied as a viable decontamination technique capable of reducing the competitive pressure exerted on MLF culture starters in freshly fermented wine.

González-Arenzana et al. tested the efficacy of PEF treatments in Tempranillo red wine at 17, 21 and 23 kV/cm (from 60 to 95 kJ/kg) in the inactivation of 25 different species of wine-associated microbiota [33]. Inactivation levels ranged from 1.70 to 3.04 log units for yeasts, from 1.01 to 4.16 for LAB, and from 0.64 to 4.94 for AAB. Similarly, Abca & Evrendilek investigated the effectivity of PEF treatments against a series of microbial strains suspended in red wine [34]. A PEF treatment at 31 kV/cm caused a reduction of more than 5.0 log cycles in the yeast population of *Saccharomyces cerevisiae*, *Hansenula anomala (Pichia anomala)*, and *Candida lipolytica*. Levels of inactivation of *Escherichia coli* and *Lactobacillus bulgaricus* with the same PEF treatment were 3.6 and 4.0 log cycles, respectively.

The application of PEF as an alternative to the addition of SO_2 in sweet wines to prevent re-fermentation was investigated by Delsart et al. [35]. A PEF treatment (20 kV/cm, 320 kJ/kg) inactivated 3.0 and 4.0 log cycles for *Saccharomyces* and *non-Saccharomyces* strains, respectively. Although the addition of SO_2 (250 mg/L) or the application of high-voltage electrical discharges (HVEDs) had a slightly greater lethal effect, PEF treatments caused less browning in the treated wines.

Attending to new consumer trends toward overall reduction of alcohol intake, wineries are producing low-alcohol wines [36]. Lower alcohol concentration might nevertheless lead to a higher risk of proliferation of spoiling or undesirable microorganisms in wine. PEF treatments (40 kV/cm, 250 μ) achieved inactivation levels up to 1.5 and 2.0 log cycles of LAB and yeasts in wines which had only 8.5% alcohol content [37].

Furthermore, a PEF treatment of 158 kJ/kg (33 kV/cm) has been validated as an improvement of the implementation of MLF starters in the production of four Tempranillo Rioja wines. The PEF-treated wines that were subjected to MLF preserved all their sensorial properties, as determined by sensory analysis through an expert panel [38].

Brettanomyces spp. is regarded as one of the most damaging and undesirable microorganisms in the wine industry due to its the high negative impact on the sensory properties of wines, even at very low concentrations. The capacity of PEF for the reduction of the population of this microorganism has been investigated by different authors. It has been observed that the lethal effect of PEF depends on the processing conditions, but differences in terms of PEF resistance among different strains have likewise been ascertained. Similar inactivation was achieved through a series of different combinations of electric field intensity and total specific energy in treatments applied under batch conditions. Inactivation of up to 4.0 log cycles was reported by applying 31 kV/cm and 150 kJ/kg [26] or 20 kV/cm and 320 kJ/kg [35]. Inactivation in the range of 2.5 to 3.0 log cycles was reported when the treatments were applied in continuous flow [33, 39].

4.3 Application of PEF for wine decontamination after malolactic fermentation

PEF inactivation of LAB strains involved in the MLF of wine has been studied by different authors. Among the microorganisms investigated, Puértolas et al. found that *Lactobacillus plantarum* and *hilgardii* displayed the highest resistance to PEF [26]. Similarly, out of a total of 25 different wine-related microorganisms,

Oenococcus oeni O46 and *Pediococcus pentosaceus* were found to be the ones most resistant to a PEF treatment (23 kV/cm, 95 kJ/kg, 49°C) [33]. PEF treatments of 20 kV/cm and 320 kJ/kg were capable of inactivating up to 5.0 log cycles of *O. oeni* with a temperature remaining below 15°C [40].

Few studies have been conducted on the inactivation of microorganisms after malolactic fermentation. González-Arenzana et al. observed that after the MLF of three wines, the application of a PEF treatment (95 kJ/kg, 23 kV/cm) in combination with a low SO_2 concentration (15 mg/L) had similar or even greater effectivity than an increased dose of SO_2 (30 mg/L) in the microbial stabilization of wine [41]. PEF treatments alone, or combined with SO_2, allowed for a significant reduction in the overall population of the main microbial strains of yeasts, LAB, and ABB. Moreover, stabilization by PEF treatments was effective in inhibiting microbial growth after six months of storage, with no changes in physicochemical and sensory properties in comparison to wines stabilized by SO_2.

4.4 Application of PEF for wine decontamination before aging in barrels

Aging in oak barrels is one of the key steps in the production of high-quality wine, due to its gradual development in terms of aroma, color, and stability [42]. Oak wood is a porous material that is necessary for air exchange and for the maintenance of low oxidation conditions in wine during the aging process, but oak wood barrels are extremely difficult to clean and sanitize. They therefore present an ideal niche for microbial proliferation, and can be a source of contamination for subsequent batches of wine [43]. This is a great concern in wineries – especially in the case of *Brettanomyces* colonization, due to that yeast's negative impact on wine quality, along with the difficulty of early identification and the considerable economic losses associated with its proliferation. Many other microbial strains can colonize the oak barrels and become a source of contamination and wine spoilage. Any strategy for the microbial decontamination of aged wine in barrels should nevertheless preserve all the quality parameters acquired during this long and expensive process.

Aged preservative-free wine in oak barrels was successfully treated by PEF, with a high-level reduction in the population of the main naturally present strains [44]. However, the recovery of some of the main microorganisms involved in aging was observed in control and PEF-treated wines after 5–9 months of storage. Therefore, different PEF parameters should be tested in order to optimize PEF conditions in this scantly investigated step of winemaking. Further studies regarding the effect of PEF treatments on valuable aging characteristics prior to bottling should be carried out, as well as on the evolution of the microbial population during these long storage periods.

5. PEF treatment effects on the physicochemical and sensory properties of wine

One of the main concerns regarding the use of preservation techniques in the wine industry lies in their potentially negative effects on the quality characteristics of wines. As a non-thermal technology, Pulsed Electric Fields presents the advantage of having great effectivity in terms of microbial decontamination with minimum alteration of the physicochemical and nutritional properties of foods [45]. A series of studies have reported that PEF has no significant effects on the main physicochemical and sensorial quality parameters of must and wine, immediately after treatment or after a period of storage [24]. What is more, some of these studies have reported better sensory attributes for PEF-decontaminated wines in comparison with untreated wines or wines treated with SO_2.

After six months of storage, the physicochemical composition of three PEF-treated wines showed no differences in pH, total acidity, anthocyanin content, or total polyphenol index, but they displayed better quality in terms of volatile acidity and color intensity [41]. Moreover, sensorial analysis indicated that the organoleptic properties of the wines treated with PEF combined with SO_2 (15 mg/L) had the highest scoring values in comparison with wines treated only with PEF or treated only with SO_2 (30 mg/L). In white wines, intense PEF treatments of 20 kV/cm and 6 ms had an effect similar to the addition of sulfur dioxide (250 mg/L), but with a notable decrease of the browning effect [35].

Moreover, the application of PEF treatments combined with mild temperatures has been proven to significantly increase microbial inactivation levels [22, 23]. In this context, Abca & Evrendilek studied changes in the attributes of wine treated by PEF combined with different temperatures for purposes of microbial inactivation [34]. For all the strains studied (*E. coli, L. bulgaricus, C. lipolytica, S. cerevisiae*, *and H. anomala*), an increment of the treatment temperature from 10 to 30°C improved the lethal effect by at least 1.5 log cycles. Even the most intense treatment (31 kV/cm, 30°C) did not show any significant changes in pH level, °Brix, titratable acidity, color, anthocyanin, antioxidant capacity, total polyphenolic content, and sensorial properties.

Until now, no study has shown any significant negative effects on the sensory properties of wine treated by PEF. Further research should nevertheless be carried out with optimized PEF-parameters for microbial stabilization in the different steps of winemaking, and featuring different grape/wine varieties.

Among potentially negative effects of PEF, another important concern is the possible migration of ion metals from the electrodes to the food matrix. Although certain authors have reported the release of ion metals, this phenomenon seems to be thoroughly dependent on electrode material and geometry, as well as on processing parameters (conductivity, electric field strength, total specific energy, pulse width) [46, 47]. In wine, the increase of certain metal ions (e.g. arsenic, calcium, mercury, iron, copper, magnesium, and selenium, among others) can cause turbidity and a metallic taste; it can even represent a health risk for consumers. In red wine, Abca & Evrendilek did not observe significant differences in the concentration of 13 different metal ions between PEF and control wines, even at highest-intensity PEF conditions (31 kV/cm, 30°C) [34]. Similarly, no differences in iron and chromium concentration were detected in Cabernet Sauvignon red wine subjected to 34 and 53 kV/cm (50us) treatments [39]. Although those treatments slightly increased the concentration of nickel in the PEF-treated wines the levels reached were below the maximum limits permitted in food products.

6. Conclusions and future perspectives

Microorganisms in winemaking are as necessary as they are undesirable, depending on the strain and/or the time it proliferates. The growth of spoilage microorganisms in must and wine not only exerts a considerable influence on consumer acceptance, but can also lead to uncountable economic losses. Currently, the spoilage of wine by microorganisms is mainly controlled by applying SO_2. However, due to the current global concern about the negative effects of SO_2 on human health, the wine industry is facing the challenge of attempting to reduce or eliminate its use. Proposed chemical or physical alternatives are insufficient or/and non-feasible for implementation as microbial stabilization procedures in the wine industry.

Pulsed Electric Fields emerge as a thoroughly suitable alternative technique for the stabilization of must and wine, or as a technique combined with low doses of SO_2 to ensure antioxidant protection. PEF efficacy has been studied against the main wine-related spoilage microorganisms along the different winemaking stages, but mostly under lab-scale or pilot plant conditions. Best results have been obtained when PEF was combined with mild treatment temperatures and/or with low concentrations of SO_2, or with other preservatives. Furthermore, several studies have reported to have found no negative effects or changes in the sensory quality of wines treated with PEF.

PEF technology is currently being applied in a number of industrial food processing applications. Thus, the development and optimization of PEF devices and chambers is accelerating in order to adapt them to current demands while facilitating the industrial implementation of such new techniques to food. The devices' flexibility has been highly improved, along with different types of treatment chambers, depending on the type of matrix and on treatment conditions.

The International Organization of Vine and Wine (OIV) recently approved the application of PEF to grapes in order to enhance and reduce maceration time in winemaking [48]. The technology is currently being evaluated as a microbial stabilization and decontamination process. The OIV resolutions thus suggest that PEF is a gentle technology without negative consequences for must or wine, but offering interesting improvements in terms of their quality. The applicability of PEF in several other winemaking steps such as maceration or aging-on-lees, along with the current feasibility of scale-up potential, makes this procedure thoroughly attractive for future implementation as a highly versatile technology in the wine industry. Furthermore, the energetic requirements for must/wine PEF-optimized pasteurization can ranged from 20 to 200 kJ/kg. Thus, the power consumptions imply very low costs in comparison with the traditional techniques and the innovative ones suggested.

Generally, however, the ranges of PEF parameters studied for purposes of microbial decontamination (at laboratory scale), are still very intense in comparison with the ones used in grape electroporation (high-intensity voltages or long treatment times). Such intense conditions have certain drawbacks for implementation in wineries due to the power limitation of current PEF devices. This implies that PEF should be applied at very low flow rates, which are not feasible in winemaking on an industrial scale. The current challenge lies therefore in studying low and mild PEF conditions not investigated so far in-depth: PEF alone, or in combination with other methods. One of the most promising combinations is the application of PEF treatments in association with mild temperatures or/and with reduced doses of SO_2. A reduced amount of studies have already proven the synergetic effect that emerges between these methods when applied in combination. Thus, in order to successfully implement PEF technology in wineries for purposes of microbial decontamination, it will be necessary to define the lowest-intensity PEF parameters which, combined with mild temperatures and reduced-SO_2, have the highest synergetic effect. This would allow for a considerable increase in the processing capacity of PEF units, thereby facilitating this technique's industrial application in the wine industry without affecting sensory properties, while attending to widespread demands for the reduction of SO_2.

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Author details

Carlota Delso, Alejandro Berzosa, Jorge Sanz, Ignacio Álvarez and Javier Raso*
Veterinary Faculty, Food Technology, Agrifood Institute of Aragón-IA2
(University of Zaragoza-CITA), Zaragoza, Spain

*Address all correspondence to: jraso@unizar.es

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Chapter 13

Influence of Skin-Contact Treatment on Aroma Profile of Malvasia *Aromatica* Wines in D.O. "Vinos de Madrid"

Julia Crespo, Valeria Romero, Margarita García, Teresa Arroyo and Juan M. Cabellos

Abstract

The effects of prefermentative cold skin-contact technique using Malvasia *aromatica* were studied as a first step to adapt to the climate change related effects in order to intensify the aroma potential of white wines of the D.O. "Vinos de Madrid" keeping the organoleptic characteristics of the region. Major volatile compounds were extracted by liquid–liquid extraction and quantified by GC-FID. Minor volatile compounds were determined by HS-SPME/GC–MS. Sensory analysis were also carried out to describe and quantify attributes of the wines. A total of 37 components were identified and quantified. Volatile components showed mixed behavior depending on the skin-contact time. Skin-contact for longer helps to enhance the floral character provided by some compounds contained in the skin, especially linalool and 2-phenyl etanol and were impact odorants of Malvasia *aromatica* wine based on odor activity values (OAVs).

Keywords: skin-contact, aroma, climate change, white wine, Malvasia *aromatica*

1. Introduction

Skin-contact treatment has been proposed as a technique to try to increase the extraction of varietal aromas from the skins in different white cultivars [1–3]. It is a technique extensively used in the production of young white wines with the aim of improving their intensity and aroma profile by transferring free and glycosidically bound aroma compounds from the grape skins to the must before fermentation begins. The compounds responsible for the varietal aromas of wines depend on grape variety, climate, and soil and will determine the quality and local character of wines. Early winemaking procedures such as skin contact and the amount of pressure applied during pressing together with temperature conditions applied, will affect the extraction of aroma compounds and their precursors into the grape juice and consequently their concentrations in the resulting wine [4–7]. In the course of maceration, the concentration of aromas may increase in the must but there are not always changes at the sensory level in the wines. The varietal characteristics of the wine may be enhanced with the skin contact, however, there is some risk of the

apparition of herbaceous aromas, bitter flavors and excessive color in the musts. For these reasons, the conditions of temperature and contact time between the skins and the juice must be carefully chosen.

The vineyard is a crop with a wide range of adaptation to different environmental and agronomic conditions whose correct development is strongly influenced by the climate. In particular, the suitability of wine-growing areas to reach optimum levels of sugar, pH, color and aromatic components, which are necessary for the production of quality wines, depends on weather conditions throughout the growing period [8, 9]. As a result, climatic fluctuations will make very difficult to produce the same kind of wine in a particular area over seasons. The wines would lose the typicity and distinction of the region being affected the local economy by the decrease of the value of the final product.

The adaptation responses to deal with climate change related effects on winemaking can be implemented at the winery level or at the vineyard level [10]. In oenology, innovations could serve to correct fluctuations in grape quality. Also, can be considered as the first strategy to protect against climate variations related effects by focusing on specific hazards in order to improve the production. These techniques include changes in winemaking practices.

Skin-contact treatment has been proposed as a first measure of adaptation to climate change related effects. This study was focused on variations skin-contact time in order to intensify the aroma potential of winemaking white wines in D.O. "Vinos de Madrid". The purpose of the present paper was to evaluate differences in white musts and wines, which would arise due to different skin-contact time using the same temperature. In particular, the aromatic and sensory characteristics of the wines. To achieve this aim we choose cv. Malvasia *aromatica*, a white grape variety of Italian origin that has been grown in Spain since the 14th century. The main characteristics of this cultivar are: from an aromatic point of view, the presence of terpenes responsible of citrus and floral aromas similar to Muscat varieties [11] and fermentation aroma compounds, mainly fatty acids and their esters, provide it with fruity aromas [3, 12, 13]. On the other hand, physical–chemical characteristics that give rise to musts with high acidity and low pH, which make it a suitable varietal for trying to improve the organoleptic quality of its white wines of D.O. "Vinos de Madrid".

2. Material and methods

2.1 Vintage

Grapes from *Vitis vinifera* L. cv. Malvasia *aromatica* were hand-collected from an experimental vineyard of the Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA), located in "Finca El Socorro" in D.O. "Vinos de Madrid", Arganda del Rey, Spain (40°8'N, 3°22'W, 715 m altitude). Final harvest time was determined when berries reached 23°Brix and transported to the Experimental Winery from IMIDRA at the "Finca El Encín", in Alcalá de Henares, Spain (40°31'N, 3°17'W, 605 m altitude).

2.2 Skin-contact treatment

After harvest, grapes were divided into two batches for each assay (1 and 2). One batch was treated in the conventional way (C) without skin-contact and was used as control. In this way grapes were crushed and pressed in a hand-press and 5 g/hl of sulfur dioxide was added. The juice was then settled at 10°C for 12–18 h,

and then racked. The total acidity in the must was corrected with tartaric acid to 6 g/L. The must was racked, dividing the volume equally in three stainless steel tanks. Commercial yeast was added for its fermentation which took place at 16°C and was followed daily by measuring density. The conventional way samples (C) of each assay (1 and 2) were different from each other, they came from different grapes.

For the skin contact treatment, the grapes were destemmed and crushed. The pomace (musts and skin) was mixed 5 g/hl of sulfur dioxide, kept at 10°C for 18 h (A1) and 6 h (A2). At the end were pressed in a hand-press (M18 and M6 assays). The juice was settled, racked and divided as mentioned in the conventional way. The rest of the process was equal to the conventional way.

2.3 Physical-chemical analysis and fermentation kinetics

Oenological parameters (°Brix, free and total sulfur dioxide, pH, total acidity, volatile acidity, ethanol (% v/v) and residual sugars) were analyzed following OIV official methods [14]. Yeast assimilable nitrogen (YAN) was determined following the Sörensen method.

A daily control of temperature and density was carried out to determine the influence of pre-fermentative skin contact on the kinetics of the fermentations. Fermentation velocity (V_F) was measured checking daily the sugar percentage lost during the fermentation. On the other hand, V_{50} amount of sugar daily transformed by the yeasts when 50% of the sugar content had been used up was also evaluated [15].

2.4 Aromatic analysis of the wines

Analysis of free aroma compounds was performed by quantification of minor and mayor volatile compounds. Quantification of major volatile compounds was undertaken by GC-FID (Agilent Technologies, Santa Clara, CA, USA) with a DB-Wax column (60 mx 0.32 mm x 0.5 m) from J&W Scientific (Folsom, CA, USA) following the procedures proposed by Ortega [16]. The liquid phase extraction (LPE) of aroma compounds was performed in dichloromethane. The method conditions were: oven temperature 40°C for 5 min, then increased to 3°C/ min up to 200°C, and helium as carrier gas at 2 ml/min. Two mL of aroma extract were injected at 250°C in splitless mode. The total run time was 75 minutes per sample. Analyses were carried out in duplicate.

Minor volatile compounds (terpenoids and C_{13}-norisoprenoids) were determined by HS-SPME/GC–MS following the method proposed by Yuan & Qian [17]. A 50/30 μm DVB/CAR/PDMS fiber (Supelco Inc., Bellefonte, PA) was used for volatile extraction. 20 mL vials were used for chromatography (Agilent Technologies). Two mL of the wine sample were diluted with 8 mL of a citric acid solution (0.5 g/L citric acid, pH 3 saturated with sodium chloride) and 20 μL of 4-octanol (100 μg/L) was used as internal standard were added with a small magnetic stir bar. The vials were capped and equilibrated at 50°C in a thermostatic bath for 10 min. The aromatic compounds were extracted through SPME fiber for 50 min at 50°C with stirring (1000 rpm). The fiber was inserted into the injection port of the GC (230°C) to desorb the compounds. The injection into the chromatograph was manual. An Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass selective detector (Agilent, Santa Clara, CA) was used. Compound separation was achieved with a DB-WAX de J&W Scientific (Folsom, CA, USA) (60 m x 0.32 mm x 0.5 μm film thickness, Phenomenex, Torrance, CA). A constant helium column

flow rate of 1.0 mL/min was used. The chromatographic program was set at 40°C for 3 min, raised to 230°C at 5°C/min for 15 min. Splitless injection mode was used.

2.5 Sensory analysis

Descriptive sensory analyses were performed by a trained panel of 8 people (4 expert tasters and 4 habitual consumers) from the IMIDRA Institute. This panel had been previously trained in the recognition of wine flavor. Sensory descriptive analysis was performed to describe and quantify attributes of the wines based on a scale from 1 (low intensity) to 10 (high intensity). A hedonic classification was also carried out establishing the order of preference of the samples presented. The final score was obtained as the mean of the wine evaluations with their respective standard deviation and interpreted by graphical representation.

2.6 Statistical analyses

The statistical processing of the data was carried out with software SPSS ver. 20.0 (SPSS, Inc., Chicago, USA). Analysis of variance (ANOVA) was applied on oenological parameters, volatile compounds and sensory attributes of the wines. Tukey HSD post-hoc tests were used to establish the significance of differences between means to assess significance ($p < 0.05$).

3. Results and discussion

3.1 General must and wine composition

General composition of must obtained with the two skin-contact treatment and conventional way from cv. Malvasia *aromatica* are given in **Table 1**. In the skin-contact treatment assays, the total acidity of the must decreases along with a slight increase in pH. This is due to the transfer of cations from the skin to the must during the previous maceration stage, and results in a decrease of acidity in the form of potassium bitartrate together with a salification of the acids [18]. The results show an increase in Yeast assimilable nitrogen (YAN) according to the time of contact with the skin, being more notable with M18; M6 did not cause variations in YAN content. These results are in agreement with the studies carried out by other authors, where a period of contact with the skin favors the enrichment of the musts in terms of amino acid content [19, 20]. In general, the effect of skin-contact in both assays is not very pronounced, which could be related to the low temperature

	A1		A2	
	C	M18	C	M6
°Brix	23.2 ± 0.1	23.1 ± 0.1	21.4 ± 0.1	20.3 ± 0.1
pH	3.20 ± 0.0	3.23 ± 0.0	3.25 ± 0.0	3.26 ± 0.0
Total acidity [a] ($g\,l^{-1}$)	5.9 ± 0.0	5.7 ± 0.0	5.7 ± 0.0	5.4 ± 0.0
YAN [b] ($mg\,l^{-1}$)	135.0 ± 0.0	151.3 ± 0.0	109.9 ± 6.4	105.0 ± 12.0

[a] *As tartaric acid.*
[b] *Yeast assimilable nitrogen (YAN).*

Table 1.
General composition of must obtained with different treatment: Conventional (C) and skin-contact treatment: Assay 1 (A1), 18 h (M18) and assay 2 (A2), 6 h (M6).

(10°C) and the time of contact, compared to other studies on white varieties (15.5°C, 20°C and 24°C [21]).

3.2 Fermentation kinetics

Figure 1 shows the average of the fermentative kinetics evolution of Malvasia musts at 10°C. Skin contact for 6 hours does not influence the development of fermentation (a), no differences were found in terms of fermentation time and velocity between vinifications (**Table 2**). However, in the case of skin-contact for 18 hours (b), there are differences in the time and velocity of fermentation compared to the conventional one. The macerated must concludes its fermentation almost a week before the conventional one. This fact may be related to the YAN content and its high content of nutrients and fermentation activators, which seem to have a strong influence on the process (see **Table 1**).

General composition of wines obtained with skin-contact treatment and conventional way from cv. Malvasia *aromatica* are given in **Table 3**. Wines from skin contact treatments had lower values for total acidity. There was no significant difference for any quality parameter that is in accordance with research published

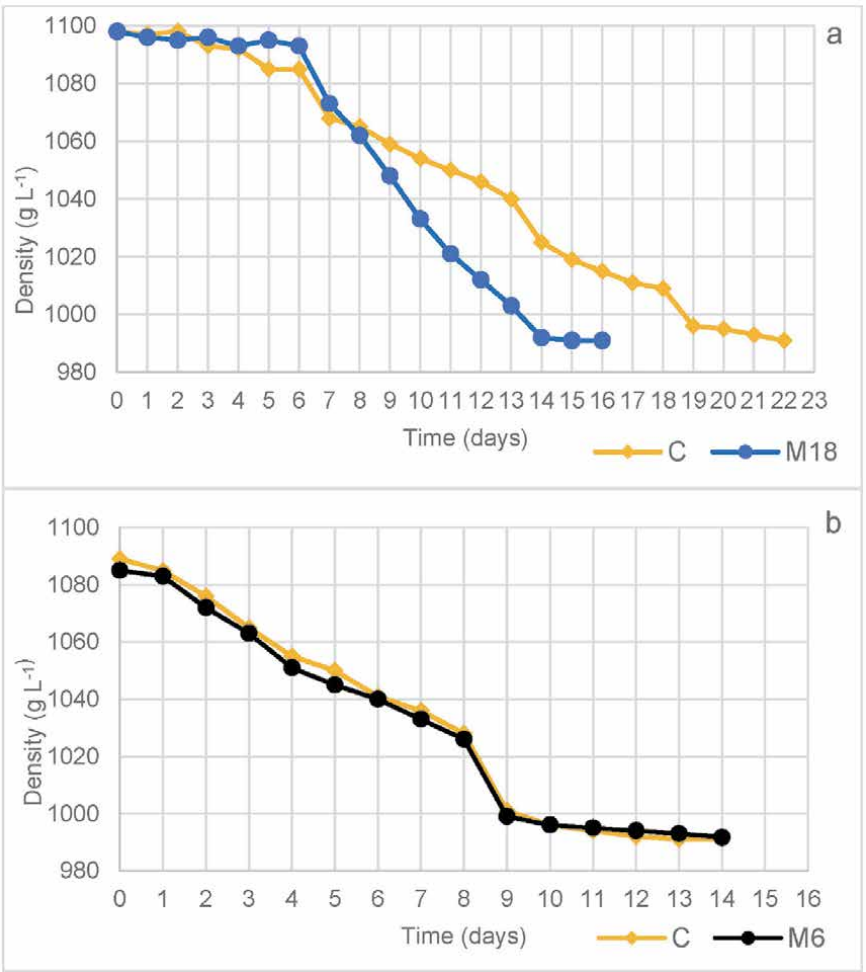


Figure 1.
Fermentative kinetics evolution of Malvasia musts. (a) Assay 1, skin-contact 18 h (M18). (b) Assay 2, skin-contact 6 h (M6).

	Treatment	V_{50} (%)	V_f (%)
A1	C	8.3	4.5
	M18	11.1	6.7
A2	C	16.7	7.7
	M6	16.7	7.1

V_{50}: amount of sugar daily transformed when 50% of the sugar content had been used up; V_f: Fermentation velocity (daily sugar % lost).

Table 2.
Influence of skin-contact on fermentation velocity.

	A1		A2	
	C	M18	C	M6
Ethanol (% v/v)	13.0 ± 0.1	12.9 ± 0.1	13.8 ± 0.1	13.0 ± 0.1
pH	3.20 ± 0.0	3.18 ± 0.0	2.95 ± 0.0	2.90 ± 0.0
Total acidity [a] ($g\ l^{-1}$)	7.1 ± 0.0	6.6 ± 0.0	6.3 ± 0.0	6.4 ± 0.0
Volatile acidity [b] ($mg\ l^{-1}$)	—	0.2 ± 0.0	0.5 ± 0.1	0.5 ± 0.0
Residual sugar ($g\ l^{-1}$)	2.8 ± 0.3	2.8 ± 0.0	1.3 ± 0.0	1.1 ± 0.1

[a] *As tartaric acid.*
[b] *As acetic acid.*

Table 3.
General composition of wines obtained with different treatment: Conventional (C) and skin-contact treatment: Assay 1 (A1), 18 h (M18) and assay 2, 6 h (M6).

studies [22–24]. As explained in point 2.2, the conventional way samples were different from each other, hence the difference in ethanol content.

3.3 Influence on aroma compounds

Varietal aromas from grapes, terpenols and C-13 and those from fermentation were determined. The aromatic compounds have been grouped by aromatic families: terpenols, C13, alcohols, lactones, acids, esters, aldehydes and ketones (**Table 4**). These were 37 aromatic compounds studied from the three processing methods together with an analysis of variance to determine the influence of two maceration times (18 hours and 6 hours) on the total volatile content. In addition, the real contribution of each compound to the aroma of the wine was measured by the corresponding perception thresholds.

Table 5 shows the odor threshold values (OTH) and their sensory descriptors for those compounds with odor activity values (OAVs) >1, which actively contribute to the aroma of the wines.

In both assays, skin contact treatment increased the total concentration of volatiles in wines compared to the control wine. From the A1, the control and M18 wines contained 303.9 and 413.9 mg/L and from A2, the control and M6 309.9 and 318.1 mg/L of volatiles, respectively. Similar results were found by other authors [6, 28] on different varieties. Also, in a study carried out using a period of contact between the skins and the must of the Narince grape variety resulted in an increase of the aromatic content of the wines subjected to maceration [29].

Higher alcohols were the most abundant family of volatile compounds in the four winemaking processes, contributing more than 90% of the total volatile

	A1			A2		
Compounds	**C**	**M18**	**Sig.[a]**	**C**	**M6**	**Sig.[a]**
Terpenols (μg l^{-1})						
β-Myrcene	1.26 ± 0.04	1.49 ± 0.18	Ns	1.03 ± 0.19	0.75 ± 0.09	*
α-Terpinene	0.22 ± 0.01	0.17 ± 0.01	Ns	0.13 ± 0.04	0.12 ± 0.03	Ns
Limonene	0.52 ± 0.03	0.36 ± 0.07	Ns	0.34 ± 0.04	0.24 ± 0.03	*
γ-Terpinene	1.59 ± 0.09	1.46 ± 0.14	Ns	1.04 ± 0.20	0.76 ± 0.11	Ns
Linalool	78.75 ± 2.25	98.12 ± 11.59	*	39.77 ± 8.67	46.83 ± 9.30	Ns
α-Terpineol	15.42 ± 1.38	17.37 ± 1.96	Ns	10.83 ± 2.43	8.83 ± 1.94	Ns
β-Citronellol	6.07 ± 0.45	26.54 ± 5.31	**	2.60 ± 0.27	4.04 ± 0.76	*
Geraniol	9.83 ± 0.25	16.03 ± 3.01	*	5.60 ± 1.15	6.00 ± 1.05	Ns
Total	*113.66 ± 2.48*	*161.55 ± 21.53*		*61.35 ± 12.13*	*67.57 ± 12.17*	
C_{13}-norisoprenoids (μg l^{-1})						
β-Damascenone	1.76 ± 0.10	0.94 ± 0.04	***	1.38 ± 0.22	1.28 ± 0.24	Ns
Total	*1.76 ± 0.10*	*0.94 ± 0.04*		*1.38 ± 0.22*	*1.28 ± 0.24*	
Alcohols (mg l^{-1})						
Isobutanol	26.81 ± 1.12	25.81 ± 2.94	Ns	14.66 ± 1.33	13.92 ± 2.03	Ns
1-Butanol	0.69 ± 0.01	0.60 ± 0.04	*	0.38 ± 0.04	0.31 ± 0.06	Ns
Isoamyl alcohol	225.17 ± 7.34	288.80 ± 29.51	*	212.63 ± 8.85	213.56 ± 25.11	Ns
1-Hexanol	1.06 ± 0.05	0.62 ± 0.24	*	0.65 ± 0.02	0.84 ± 0.08	Ns
Cis-3-hexen-1-ol	0.47 ± 0.03	0.28 ± 0.11	*	Tr	Tr	
Methionol	1.23 ± 0.16	3.28 ± 0.66	**	0.80 ± 0.06	0.81 ± 0.13	Ns
Bencylalcohol	0.29 ± 0.03	0.03 ± 0.00	Ns	0.00 ± 0.00	0.00 ± 0.00	Ns
2-Phenylethyl alcohol	33.49 ± 5.20	75.14 ± 26.35	*	56.30 ± 6.99	60.55 ± 7.36	Ns
Total	*289.20 ± 5.06*	*394.56 ± 51.10*		*285.42 ± 6.51*	*289.99 ± 34.74*	
Lactones (mg l^{-1})						
y-Butyrolactone	0.49 ± 0.10	0.91 ± 0.14	*	1.67 ± 0.22	2.53 ± 0.37	*
Total	*0.49 ± 0.10*	*0.91 ± 0.14*		*1.67 ± 0.22*	*2.53 ± 0.37*	
Fatty acids (mg l^{-1})						
Isobutyric acid	0.63 ± 0.03	0.27 ± 0.01	*	2.26 ± 0.10	1.99 ± 0.20	Ns
Butyric acid	Tr	Tr		0.24 ± 0.01	0.26 ± 0.02	Ns
Isovaleric acid	0.89 ± 0.04	2.06 ± 0.99	Ns	2.88 ± 0.16	2.81 ± 0.23	Ns
Hexanoic acid	2.11 ± 0.39	1.49 ± 0.47	Ns	2.95 ± 0.21	3.68 ± 0.43	*
Octanoic acid	1.85 ± 0.30	1.27 ± 0.25	*	4.35 ± 0.25	6.34 ± 0.99	*
Decanoic acid	0.14 ± 0.01	0.14 ± 0.00	Ns	0.43 ± 0.02	0.63 ± 0.02	*
Total	*5.62 ± 0.67*	*5.22 ± 0.64*		*13.11 ± 0.60*	*15.70 ± 1.85*	
Esters (mg l^{-1})						
Ethyl butirate	0.19 ± 0.02	0.10 ± 0.00	**	0.67 ± 0.04	0.53 ± 0.07	Ns
Ethyl isovalerate	0.46 ± 0.02	0.38 ± 0.08	Ns	0.33 ± 0.04	0.41 ± 0.08	Ns
Isoamyl acetate	0.76 ± 0.07	0.54 ± 0.10	*	4.11 ± 0.68	4.58 ± 0.44	Ns
Ethyl hexanoate	0.29 ± 0.02	0.74 ± 0.13	*	0.40 ± 0.04	0.47 ± 0.06	Ns

	A1			A2		
Compounds	**C**	**M18**	**Sig.[a]**	**C**	**M6**	**Sig.[a]**
Hexyl acetate	0.08 ± 0.02	0.03 ± 0.00	*	0.10 ± 0.01	0.12 ± 0.02	Ns
Ethyl lactate	1.24 ± 0.03	0.03 ± 0.00	Ns	1.46 ± 0.05	1.56 ± 0.14	Ns
Ethyl octanoate	0.15 ± 0.00	0.15 ± 0.00	Ns	0.72 ± 0.09	0.73 ± 0.13	Ns
Ethyl 3-Hidroxy-butirate	0.11 ± 0.01	0.17 ± 0.03	*	0.18 ± 0.02	0.19 ± 0.02	Ns
Diethyl succinate	0.24 ± 0.05	0.18 ± 0.00	Ns	0.11 ± 0.01	0.08 ± 0.02	Ns
2-Phenylethyl acetate	1.55 ± 0.17	1.72 ± 0.32	Ns	0.79 ± 0.07	0.89 ± 0.11	Ns
Total	*5.07 ± 0.18*	*4.03 ± 0.31*		*8.87 ± 0.87*	*9.56 ± 0.69*	
Carbonyl compounds (mg l^{-1})						
Diacetyl	Tr	Nd		0.32 ± 0.02	0.23 ± 0.02	**
Acetoín	3.42 ± 0.20	8.73 ± 2.03	*	Tr	Tr	
Benzaldehíde	Tr	0.29 ± 0.01	***	Tr	Tr	
Total	*3.42 ± 0.20*	*9.02 ± 2.03*		*0.32 ± 0.02*	*0.23 ± 0.02*	
Total (mg l^{-1})	***303.91 ± 5.72***	***413.91 ± 49.25***		***309.45 ± 32.54***	***318.07 ± 37.61***	

*[a]Significance at which means differ as shown by analysis of variance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Ns: not significant; Nd: non detected; Tr: traces.*

Table 4.
Effect of skin contact on the aroma compound levels of Malvasia aromatica *wines.*

content analyzed. Higher alcohols, in quantities below 300 mg/L can contribute to improving the aromatic complexity of white wines, however are considered to be a negative factor in terms of aromatic quality when they exceed 400 mg/l [30]. Isobutanol, isoamyl alcohol and 2-phenylethanol were the most abundant in the four wines analyzed. Among the higher alcohols, M18 has increased the levels of 2-phenylethanol being 5.3 (**Table 5**). This compound is related to floral aromas with attributes of roses and is considered to contribute positively to wine aroma [31]. There has been a significant decrease of 1-hexanol y cis-3-hexen-1-ol in M18 wines in comparison to the control. These compounds are related to herbaceous aromas and bitter taste so are unfavorable to wine quality. Skin contact treatment for 18 h resulted in significant increase in the concentration of the esters ethyl 3-hydroxybutyrate and ethyl hexanoate esters, however, the concentrations of ethyl butyrate, isoamyl acetate and hexyl acetate decreased with the maceration time. Esters are very important for the aroma of wine, they are related to fruity aromas [32]. Due to their high OAVs (**Table 5**), ethyl butyrate (apple), ethyl isovalerate (orange), isoamyl acetate (banana), ethyl hexanoate (green apple) and 2-phenylethyl acetate (flowers) should be considered as important contributors to the typical aroma of Malvasia wines. In the case of M6 no differences were found on any of the esters studied so we can conclude that maceration for a reduced period of time has not affected the ester content of the resulting wines.

Eight terpenes were identified in the wines, among them, linalool, β-citronelol and geraniol increased significantly with M18 while with M6 only β-citronelol increased significantly. Ninety percent of geraniol is in the skins, while linalool is distributed 50% between the skin and 50% in the pulp [33, 34]. Other authors [35] reported high concentrations of geraniol and its derived products throughout the ripening process in Malvasia grapes. Only linalool reached concentrations above its odor threshold in all wines, with the highest significant extraction in M18 wines.

	Sensory descriptor	OTH*	[a] OAV			
			C	M18	C	M6
Linalool	Floral, citric	25[b]	3.15	3.92	1.59	1.87
β-damascenone	Floral, lilac	0.05[c]	35.20	18.70	27.50	25.60
Isoamyl alcohol	Bitter	30[b]	7.50	9.63	7.09	7.12
Cis-3-hexen-1-ol	Herbaceus	0.4[b]	1.10			
Methionol	Onion, cauliflower	1[b]	1.20	3.30		
2-Phenylethyl alcohol	Roses	14[b]	2.40	5.30	4.02	4.32
Butyric acid	Cheese	0.17[b]				1.50
Isovaleric acid	Blue cheese	0.03[b]	29.60	68.60	96.00	93.60
Hexanoic acid	Cheese	0.42[b]	5.00	3.50	7.00	8.80
Octanoic acid	Butter, sour	0.50[b]	3.70	2.50	8.70	12.70
Ethyl butirate	Acid fruit, apple	0.02[b]	9.47	1.90	33.57	26.62
Ethyl isovalerato	Sweet fruit, orange, blackberry	0.003[b]	152.11	127.01	109.63	135.77
Isoamyl acetate	Banana	0.03[b]	25.46	18.00	136.85	152.58
Ethyl hexanoate	Fruit, Green apple	0.01[b]	29.37	73.71	39.67	46.91
Ethyl octanoate	Fruit, grapefruit	0.58[c]			1.24	1.25
2-Phenylethyl acetate	Floral, honey	0.25[b]	6.20	6.89	3.18	3.58
Diacetyl	Butter	0.10[c]			3.18	2.26

**OTH: Odor threshold values.*
[a]OAV: Odor activity values calculated by dividing concentration by odor threshold value of the compound. OTH and OAV are given in mg l^{-1} except linalool and β-damascenone which are in μg l^{-1}. Sensory descriptor according to:
[b][25, 26].
[c][27].

Table 5.
Odor threshold values and odor activity values of the volatile compounds with the greatest influence on the aroma of Malvasia wines from the two skin contact treatment (A1: C-M18; A2: C-M6).

This terpene gives the wine floral and citrus notes (**Table 5**) typical of Muscat because it is one of the main compounds involved in the typical aromas of this variety [36]. Similar results were found by other authors in wines from white varieties for this family of compounds [23, 37].

β-damascenone was the only compound from the C13-norisoprenoid family found in Malvasia wines. The concentration of this compound decreases with skin-contact time, showing a significant decrease in M18 wine. C13 come from the carotenoids degradation and the hydrolysis of their glycosylated forms. In young wines they are usually present in the form of glycoconjugates [38, 39]. According to the OAVs, in all wines β-damascenone is above its perception threshold and should be considered as an important compound in the aroma of Malvasia wines (**Table 5**). Provides floral aromas with lilac attributes [17]. Other authors agree with these results for this variety [40].

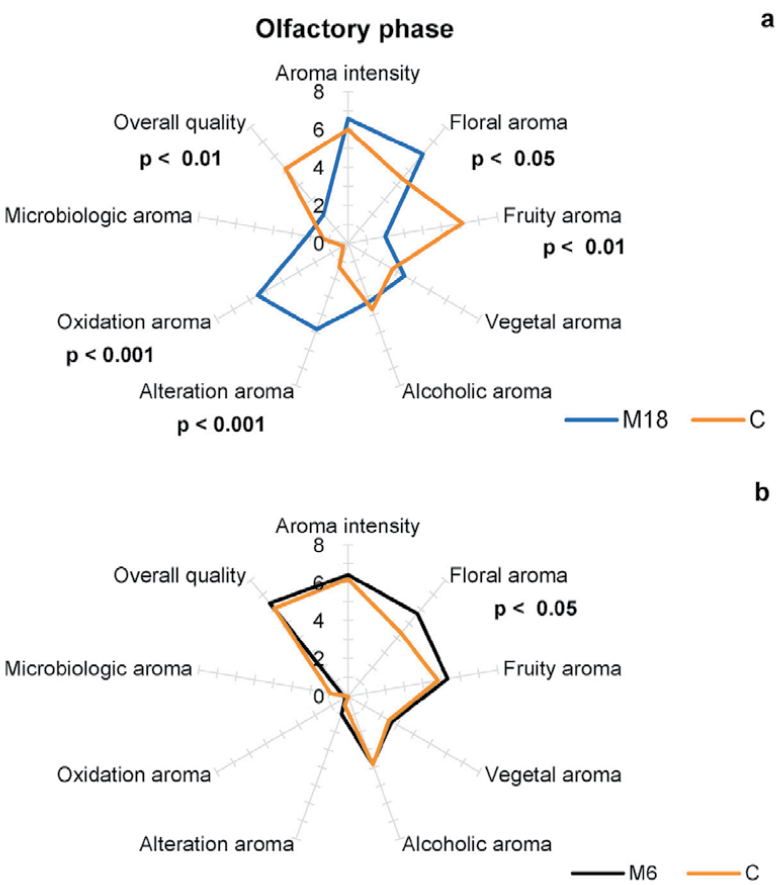
The most abundant fatty acids in the wines were hexanoic and octanoic acid (**Table 4**). These results are in agreement with those found by other authors [3, 41, 42]. The maceration seems to have different effects depending on the compound and the contact time between the skin and the must. In the case of M18 wines, the total concentration of fatty acids decreases, being particularly significant in octanoic acid. In M6 wines the total concentration of fatty acids increased significantly for hexanoic,

octanoic and decanoic acid regards to the conventional way. In all wines, regardless of the increase or decrease produced as a result of skin-contact, isovaleric, hexanoic and octanoic acids have OAVs >1 so must to be accounted in the aroma of Malvasia wines (**Table 5**). Regarding the group of aldehydes and ketones, it is known that alterations due to oxidation processes, imply the appearance of unpleasant aromas (cooked vegetables) related to the presence of compounds such as benzaldehyde, acetoin, hexanal, methional etc. [43]. Acetoin and benzaldehyde were detected in the control and M18 wines, with a significant increase in both with the maceration process ($p < 0.05$ and $p < 0.001$ respectively). According to [44] on the Verdejo grape variety, the presence of acetoin in white wines is considered negative for the flavor. In both cases, acetoin and benzaldehyde concentrations are below their perception threshold 150 mg/L [45] and 5 mg/L [46].

The two treatments (M18 and M6) significantly increased the concentration of γ-butyrolactone respect to the conventional way but in all cases it was far from its OTH (35 mg/L [47]).

3.4 Influence on sensory profile of wines

Wines were evaluated using descriptive and preference tests. The olfactory phase of the Malvasia wines from assay 1 (A) and assay 2 (B) is shown in **Figure 2**. The macerated wine (a) 18 hours had a higher score in the descriptors of altered



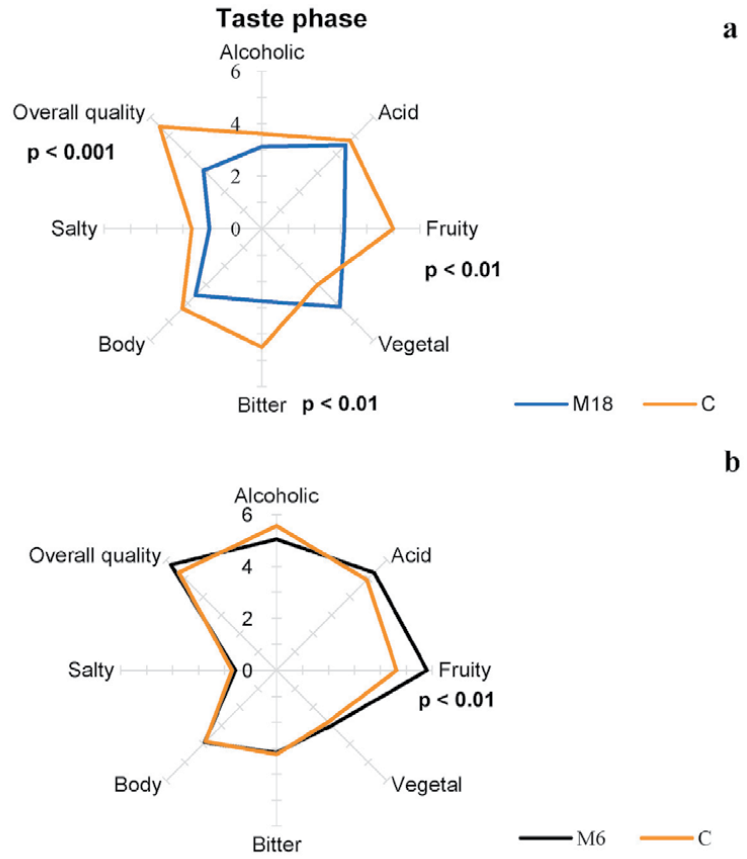
Tukey test with significance at which means differ as shown by analysis of variance: $p < 0.05$; $p < 0.01$; $p < 0.001$.

Figure 2.
Olfactory phase for the sensory analysis of the Malvasia wines from assay 1 (a) and assay 2 (b).

aroma due to problems during the conservation process of the M18 wine. Tasters also indicated oxidation aromas in M18 sample with a significance level of $p < 0.001$. The conventional wine in assay 1 was scored positively on overall aroma quality and fruity character ($p < 0.01$). In spite of the above-mentioned defects, the M18 wine received the highest score in floral character, being significantly superior to the control wine. This fact is in consonance with the results obtained in the aroma profile of these wines (see **Table 4**). In **Figure 2(b)**, M6 wines score higher in terms of fruit and floral aromatic intensity ($p < 0.05$). The rest of the parameters obtained similar scores regarding their control.

Figure 3 contain graphs of the taste using different winemaking methods. The results of the taste evaluation of in assay 1 (a), show significant differences in favor of C wine in overall taste quality ($p < 0.001$), bitterness ($p < 0.01$) and fruity character ($p < 0.01$). This could be related to the oxidation suffered by the M18. In case of assay 2, M6 wine (b) received the highest score in the fruity character with respect to the control ($p < 0.01$). This fact may be related to the release of varietal aromas through the hydrolysis of aromatic precursors by the enzymatic activity over the period of conservation in the bottle.

In the preference test, in A1 the preferences were shared between the M18 and C wines. The most preferred wine was the one produced with a 6 h skin contact treatment in the A2.



Tukey test with significance at which means differ as shown by analysis of variance: $p < 0.05$; $p < 0.01$; $p < 0.001$.

Figure 3.
Taste phase for the sensory analysis of the Malvasia wines from assay 1 (a) and assay 2 (b).

4. Conclusions

This first study in order to combat climate change related effects, the aromatic profile of Malvasia wines winemaking with different skin-contact time shows some relevant conclusions. Volatile components showed mixed behavior depending on the skin-contact time. Some compounds increased in concentration with time, while others decreased. Skin-contact for longer helps to enhance the floral character provided by the terpenols contained in the skin, especially linalool, major alcohols such a 2-phenylethanol. It also helps the increase of some esters (ethyl 3-hydroxy butyrate, ethyl hexanoate and 2-phenylethyl acetate) and the loss of others (isoamyl acetate, ethyl isovalerate and ethyl butyrate), all related to the fruity character of the wines. Short skin-contact does not cause significant effect on the content of terpenols, or ester content. The β-damascenone remains constant during M6 period, on the contrary, decreases significantly in case of M18. In general, the results of the sensory analysis show a preference for wines macerated for 6 hours. The wines macerated for 18 hours highlighted their floral character. The skin-contact process needs more studies at different time periods to optimize the aromatic potential of the grape and wine and oenological and conservation conditions of the wine.

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

D.O.	Origin Appellation
YAN	Yeast assimilable nitrogen
DVB/CAR/PDMS	divinylbenzene/carboxen polydimethylsiloxane
GC-FID	gas chromatography with flame-ionization detection
GC/MS	gas chromatography–mass spectrometry
LPE	liquid phase extraction
HS-SPME	Headspace-solid phase microextraction
C13	C13-norisoprenoids

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Author details

Julia Crespo*, Valeria Romero, Margarita García, Teresa Arroyo and Juan M. Cabellos
Department of Agrifood Research, Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA), Finca "El Encín", Alcalá de Henares, Madrid, Spain

*Address all correspondence to: julia.crespo.garcia@madrid.org

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Chapter 14

Winemaking in Cold Regions with Buried Viticulture in China

Ma Tengzhen and Han Shunyu

Abstract

China has a long history of grape cultivation and wine making, and it has grown to be one of the most important countries in terms of grape cultivation, wine production, and wine consumption. According to meteorological and geographical regionalization, China's wine production area has been divided into 11 regions, the majority of which are located in cold and mid-temperate regions in northern China, where vines must be buried in winter and unearthed in spring. In China, the main cultivated grape varieties are similar, with the red variety accounting for more than 80% of the total, while the white variety represents just 20%. Currently, Cabernet Sauvignon is the most widely planted variety, but Marselan, another red variety, have recently shown good prospects. Wild grape species such as *Vitis amurensis*, *Vitis davidii*, and *Vitis quinquangularis* are widely planted in northern and southern China because of their good resistance to local climate. This chapter highlights some common wild grape varieties in China, as well as the wines made from them. Also, some winemaking pretreatment techniques are reported.

Keywords: wine, China regions, buried viticulture, wild species, pretreatment technics

1. Introduction

China has an ancient history of beverage making. A fermented beverage of rice, honey, and fruit (hawthorn fruit and/or grape) absorbed into pottery jars from the early Neolithic village of Jiahu in China's Henan province indicate the beverage's earlier existence, dated back to 7000 B.C [1]. The viticulture and enology history in China could be traced back to the Han dynasty (138 B.C.). Zhang Qian was the first to introduce vines and winemaking techniques into China through the Silk Road. Since then, wine has been made in all of ancient China's dynasties [2], although it did not become popular until the Tang dynasty (618–907 A.D.). As a symbol of Chinese wine culture, many famous poetries were written and spread for thousands of years. During the Yuan dynasty (1271–1368 A.D.), the government instructed wine and other fruit beverages to be a replacement for cereal grain beverages. Moreover, an agricultural science literature known as 'Nong Sang Ji Yao' also recorded viticultural and winemaking practices in detail, which formed the most prosperous period of the wine industry in ancient China's history. The modern Chinese wine industry began at the end of the 19th Century when a high-ranking official brought more than 100 *Vitis vinifera* vines from Europe, and the first winery Changyu was established in Shandong province in 1892, which still holds the

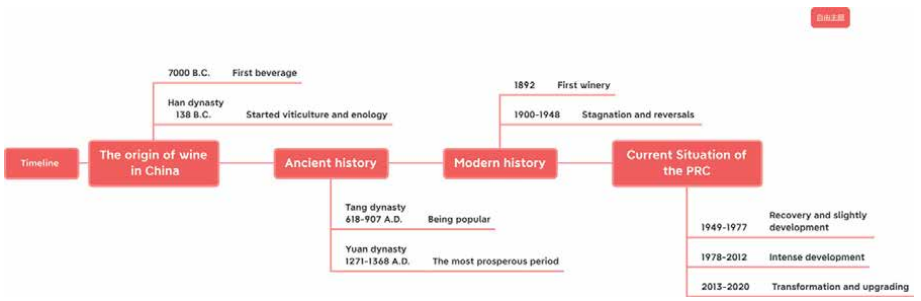

Figure 1.
Summary of the history and development of China wine industry.

leading position in Chinese wine today. With the birth of the People's Republic of China (PRC) in 1949, the Chinese government became heavily involved in the country's wine industry, expanding vineyard areas, wineries, and wine production. The contemporary wine industry underwent recuperation and considerable development at this time, but it was not until the reform and opening-up policy in 1978 that wine output increased substantially [3]. After decades of rapid growth, total wine production decreased year by year beginning in 2013, but both import volume and total wine consumption increased, indicating that China's wine market is still expanding (**Figure 1**). As one of the biggest and dynamic international markets, wines from all over the world gathered, competed, traded, and merged, causing China's wine industry to progress and upgrade over and over again. Despite this, opportunities and challenges coexisted in such a market [2].

2. Grape and wine industry in China

In the past decades, the area used for grape cultivation and the total wine production and consumption in China has rapidly expanded. Relevant statistics regarding the grape and wine industry since the birth of the People's Republic of China are shown in **Table 1**.

As can be seen from **Table 1** below, China has accomplished great success in the grape and wine industry with unprecedented speed, both in terms of vineyard area, wine production, and consumption. According to the latest International Organization of Vine and Wine (OIV) report on the world Viti vinicultural situation (2019 and 2020) [4, 5], the size of the total world area under vines (regardless of the final destination of the grapes and including vineyards not yet in production) remained stable at 7.3 mha (millions of hectares) in 2020. With 961 kha, Spain remains the clear leader in terms of cultivated vine area, followed by France (797 kha) and China (785kha).

The world wine production (excluding juice and musts) in 2020 was estimated at 258 mhl as Italy (49.10 mhl) maintained its position as the world's leading producer, followed by France (46.60 mhl) and Spain (40.70 mhl). China, on the other hand, produced 6.60 mhl. The data shows a slight drop in global wine consumption (estimated at around 234 mhl) in 2020 because of the COVID-19 outbreak. The United States (33.0 mhl), France (24.7mhl), and Italy (24.5 mhl) maintained their top three positions as the world's largest consuming countries with China ranking sixth with 12.4 mhl consumption in the world.

In China, Red varieties account for nearly 80% of the total vineyard area, while the white varieties proportion was only 20% [3]. Red wine is also far more popular

Year	Vineyard area (kha)	Grape production (mt)	Wine production (mhl)	Year	Vineyard area (kha)	Grape production (mt)	Wine production (mhl)
1950	3.2	0.04	0.83(khl)	2000	283	3.28	2.02
1959	18	0.09	0.08	2005	408	5.79	4.34
1965	11.5	0.1	0.12	2010	513	8.14	10.89
1970	/	0.09	0.2	2012	613	10.01	13.82
1975	64	0.12	0.35	2014	689	11.73	11.61
1980	32	0.11	0.78	2016	713	12.63	11.37
1985	87	0.36	2.33	2018	820	13.67	6.29
1990	121	0.86	2.54	2020	785	14.2	6.6
1995	149	1.74	2.29				

Note: *It is estimated that wine grape production area only occupies 10% of the total vineyard.*
Units: *kha, thousands of hectares; mt, millions of tons; khl, thousands of hectolitres; and mhl, millions of hectolitres.*
Source: *National Bureau of Statistics in China (vineyard area and grape production), and China alcoholic drinks association (wine production).*

Table 1.
The vineyard area, grape production, and wine production in China.

in the Chinese market than other types of wine, and a large section of the population refers to such wine as "红酒" (Hóngjiǔ), because of its red color.

3. General climatic and agronomic conditions of wine regions in China

According to administrative division and the meteorological and geographical regionalization, China wine producing regions have been widely categorized into 11 recognized regions [6], including the Northeast, the Eastern Region of Helan Mountain, Beijing-Tianjin-Hebei (also known as Jing-Jin-Ji), Shandong (also known as Jiaodong Peninsula), Old Course of the Yellow River, Loess Plateau, Inner Mongolia, Hexi Corridor, Southwest Alpine, Xinjiang and Others (**Figure 2**).

As can be seen from **Figure 2**, viticulture and enology are widely distributed in China, from 24 to 47°N, 76–132°E. The majority of vineyards are located in northern China, where they are affected by the continental monsoon climate with cold, dry winters and extremely low temperatures of −15°C during the winter. The fatal flaw for grape varieties is not only extremely low temperatures but also large amounts of water evaporation caused by extreme droughts in spring and winter, often known as 'drought-freezing'. As a result, measures have been adopted to protect vines from the cold and drought during the winter months. One of the most effective methods is to bury the vine in the soil, which is also known as buried viticulture.

In addition, some sub-areas in China's south and southwest have been identified as wine producing regions. These regions are generally located at a high altitude with a complex ecological condition, also suitable for the cultivation of *Vitis vinifera* species. However, the most planted grapes are traditional Chinese varieties such as *Vitis quinquangularis* and *Vitis heyneana* as well as their hybrid varieties (**Table 2**). The detailed information of China wine production regions, including the location, latitude & longitude, vineyard area (kha), main variety, wine production volume (mhl), meteorology, climatic subdivisions, altitude (m), and agrotype are shown in **Table 2**.

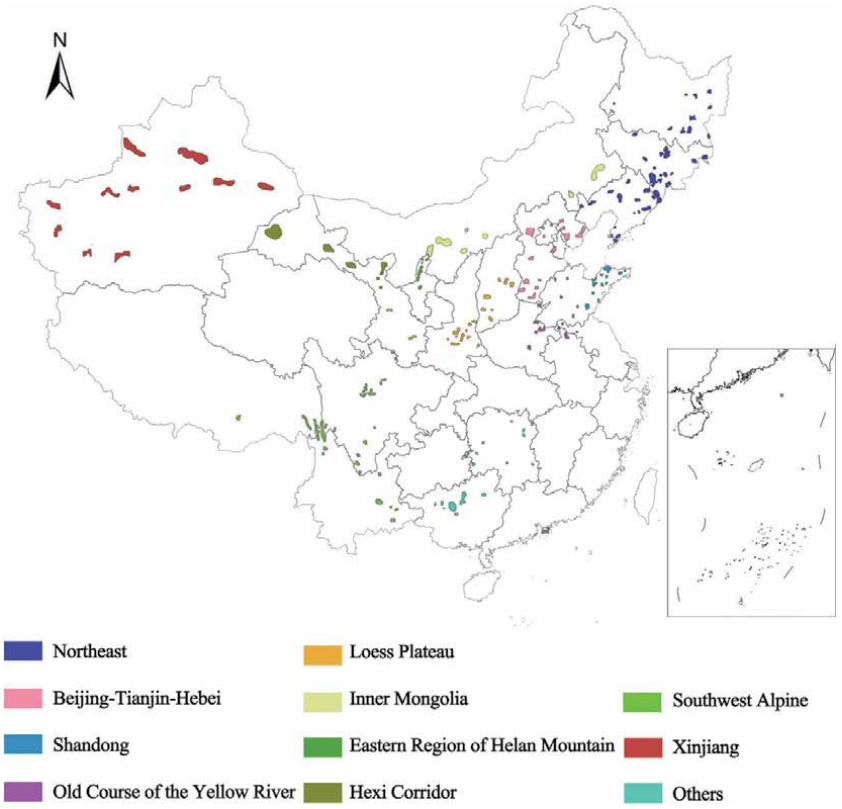

Figure 2.
Chinese wine production regions.

The vineyard area for wine grape in each region can be seen from **Table 2**, with a total of 163.39 kha, however, the CADA report (2018) shows that the wine grape area in China was only 85.19 kha, which could be due to some table grapes that are also used for winemaking being counted in **Table 2**.

In China, the main cultivated grape varieties in most regions are similar. The red grape varieties play a dominant role which occupies more than 80% [3], and among them, Cabernet Sauvignon is the most widely planted variety, followed by Merlot and Cabernet Gernischt (**Table 2**).

Recently, a new red variety, *Vitis vinifera* L.cv. Marselan, which was bred in 1961 by the French National Institute for Agricultural Research (INRA), and introduced in China in 2001, showed good adaptability in China and was considered a new star variety in China wine regions. The parent variety of Marselan is two famous red grape varieties, Grenache and Cabernet Sauvignon. Wines made from Marselan showed both parent characters, with medium-bodied and fine tannins, good color, intense fruity aroma presented in cherry and cassis flavor [8]. Nowadays, Marselan is being planted in Hebei, Shandong, Xinjiang, Ningxia, and Gansu Regions. Some wineries made wines from the single or blended Marselan variety and won lots of important awards. According to some domestic experts, Marselan wine is well suited for Chinese consumers and could be a very potent variety in China.

White grape varieties only represent a small quantity of about 20% in China. Among them, Chardonnay, Italian Riesling, and Riesling are the commonly cultivated varieties in the various regions (**Table 2**). A traditional white grape variety known as Longyan, has the potential to be utilized as both a table grape and a wine grape. As a late-harvested variety, the Longyan grape has been widely cultivated in

Regions	Producing area	Latitude & Longitude	Vineyard area (kha)	Main variety	Wine production (mhl)	Frost-free period (d)		Rainfall mm	Drought index	Climatic subdivisions	Active accumulated temperature (>10°C)	Extreme low temperature °C	Altitude (m)		Agrotype
						Range	Average value						Range	Average value	
Northeast	Jilin, Liaoning, Heilongjiang	39°18′-45° 45′N, 118° 50′-133° 30′E	8.25	*Vitis amurensis* and its hybrid variety: Gongniang No.1, Shuang, Hong, Shuang You, Zuo You Hong, Bei Bing Hong, Gong Zhu Bai, Vidal	1.15	147–222	171	400–1000	0.67–1.61	Cold temperate and mid-temperate semi-humid region	2567–2779	−33.7 ~ −15	12.2–422	207.15	Chernozems
Beijing-Tianjin-Heibei	Changli, Tianjin, Huaizhuo Basin	36°03′-42° 40′N, 113° 27′-119° 50′ E	17.01	Cabernet Sauvignon, Cabernet Gernischt, Melort, Muscat Hamburg, Chardonnay, Italian Riesling, Longyan	0.72	162–228	206	350 ~ 770	0.85–2.26	Warm-temperate semi-arid to semi-humid region	3800–4200	−23.4 ~ −14.2	1.30–629.30	190.78	Cinnamon soil, Fluvo-aquic soil, Brown earth
Shangdong	Jiaodong Peninsula, Central Shandong, Northwestern Shandong, Southern Shangdong	34°22′-38° 23′ N, 114° 47′-122° 43′ E	16.75	Cabernet Sauvignon, Cabernet Gernischt, Melort, Cabernet Franc, Chardonnay, Italian Riesling	3.84	212–241	230	550–950	0.81–1.55	Warm-temperate semi-humid region	3800–4600	−15.3 ~ −10.2	4.80–171.5	68.6	Brown earth

Regions	Producing area	Latitude & Longitude	Vineyard area (kha)	Main variety	Wine production (mhl)	Frost-free period (d)		Rainfall mm	Drought index	Climatic subdivisions	Active accumulated temperature (>10°C)	Extreme low temperature °C	Altitude (m)		Agrotype
						Range	Average value						Range	Average value	
Old Course of the Yellow River	Henan, Anhui, Jiangsu	33°36′-34° 56′N, 114° 49′-117° 12′ E	1.5	Cabernet Sauvignon, Melort, Cabernet Franc, Chardonnay, Italian Riesling, Rkatsiteli, Bacco Noir	1.88	228–245	238	600 ~ 900	0.91–1.25	Warm-temperate semi-humid region	4000	−11.6 ~ −9.78	34.7–110.4	57.8	Yellow moist soil
Loess Plateau	Shanbei plateau, Kuan-Chung Plain, Qinling-Daba Mountain, Central Shanxi, Southern Shangxi	33°21′-39° 35′ N, 107° 59′-113° 01′E	3.74	Cabernet Sauvignon, Melort, Cabernet Gernischt, Yan 73, Meili, Chardonnay, Ugni Blanc, Italian Riesling, Ecolly Bei Bing Hong, Hu Tai	0.34	165–254	213	300 ~ 700	1.19–2.09	Mid-temperate and warm-temperate semi-arid to semi-humid region	3000–4500	−23.5 ~ −8.6	402.9–1134.6	654.2	Black loessial soil, Cultivated loessial soil, Yellow-brown earth, Cinnamon soil
Inner Mongolia	Wuhai	39°15′-39° 52′ N, 106°36′-107°06′ E	6.14	Cabernet Sauvignon, *Vitis amurensis*, Beibinghong	0.03	143–184	169	50–450	1.50–6.91	Cold and mid-temperate arid to semi-arid region	2800–3600	−26.0 ~ −20.2	178.7–1561.4	911.6	Sandy loam soil, Loamy soil, Gravelly soil
Eastern Region of Ningxia Helan Mountain	Yinchuan, Qingtongxia, Hongsibu, Yongning, Helen	37°28′-39° 05′ N, 105°21′-106°80′ E	34	Cabernet Sauvignon, Melort, Cabernet Gernischt, Cabernet Franc, Pinot Noir Chardonnay,	0.34	172–190	183	200–700	4.31–5.22	Cold and mid-temperate arid region	3100–3500	−21.2 ~ −18.9	1092.5–1128.8	1110.9	Sierozems, Eolian sandy soil, Cumulated irrigated soil

Regions	Producing area	Latitude & Longitude	Vineyard area (kha)	Main variety	Wine production (mhl)	Frost-free period (d)		Rainfall mm	Drought index	Climatic subdivisions	Active accumulated temperature (>10°C)	Extreme low temperature °C	Altitude (m)		Agrotype
						Range	Average value						Range	Average value	
				Italian Riesling, Riesling											
Hexi Corridor	Wuwei, Zhangye, Jiayuguan	36°46′-40°12′ N, 93°99′-104°43′ E	20.55	Cabernet Sauvignon, Pinot Noir, Melort, Cabernet Gernischt, Chardonnay, Italian Riesling, Vidal *Vitis amurensis*	0.82	141–213	173	37.3–230	2.22–31.42	Cold temperate arid to semi-arid region	3200	−22.7 ~ −14.4	11390–2311.8	1517	Gravelly soil, Sandy loam soil
Xinjiang	North Slope of Tianshan Mountains, Lli Valley, Yanqi Basin, Turpan-Hami Basin	39°30′-44°10′ N, 80°28′-96°23′ E	36.7	Cabernet Sauvignon, Melort, Yan 73, Marselan, Syrah Chardonnay, Riesling, Pitit manseng,	0.52	176–242	199	50 ~ 300	3.91–246.45	Mid-temperate arid region	3500–4000	−31.9 ~ −13.6	1.0–1422.0	837.6	Brown desery soil, Gray desery soil, Fluvo-aquic soil
Southwest Alpine	Southwest Sichuan, Western Sichuan Plateau, Shangri-La region, Southeast Yunnan	23°50′-31°43′N, 99°70′-103°49′ E	5.45	Cabernet Sauvignon, Melort, Cabernet Gernischt, Fa-guoyeRose HoneyCrystal	0.31	278–353	273	500 ~ 800	0.66–1.92	Subtropical semi-humid region	3000–5000	−10.6 ~ −0.3	1254.1–3319.0	1986.3	Gravelly sandy loam, Cinnamon soil, Red earth, Lime soil, Brown earth, Red clay soil, Cinnamon soil, Torrid red soil, Sandy soil

Regions	Producing area	Latitude & Longitude	Vineyard area (kha)	Main variety	Wine production (mhl)	Frost-free period (d)		Rainfall mm	Drought index	Climatic subdivisions	Active accumulated temperature (>10°C)	Extreme low temperature °C	Altitude (m)		Agrotype
						Range	Average value						Range	Average value	
Others	Northern Hunan, Southeastern Hunan, Hechi	23°47′-29° 57′ N, 108° 47′-113°77′ E	13.3	Vitis *davidii*: Ziqiu, Xiangniang No.1, Vitis *quinquangularis*: Yeniang No.1, Yenaing No.2	0.07	277–365	314		0.44–0.72	Subtropical humid region	>5000	−5.0 ~ −3.6	40.2–355.5	218.6	Red earth, Yellow earth, Lateritic red earth, Humid-thermo ferralitic

Source: *Adapted from Li [6] and Sun [7].*

Table 2.
A detailed description of China wine regions.

Beijing-Tianjin-Heibei, Shandong, and Loess Plateau regions for the development of wine characterized by a green to yellow color, fresh fruity flavor, and good taste [8].

4. Wild grape species and the elaborated wine in China

China has very abundant *Vitis germplasms* in diverse species, which are distributed extensively within the country. Some Chinese wild grape species, *Vitis davidii*, *Vitis quinquangularis*, and *Vitis amurensis*, which have a long history of use in China, were widely planted to support the domestic grape and wine industry as these species showed strong environmental adaptability to the local climate [9]. In many parts of China, the fruit of Vitis wild species has been employed in winemaking whereby wines made from these grapes have a distinctive color, aroma, and taste, quite unlike those made from *Vitis vinifera* [10].

Vitis amurensis and its hybrid varieties are the most important in the Northeast due to their ability to withstand the cold winters, whereas *Vitis davidii* and *Vitis quinquangularis* are widely cultivated in the Southwest Alpine and Other regions due to their ability to withstand the high temperatures and humidity in southern China. The fruit berry characters of these Vitis wild species are similar, with low content of sugar, high content of acids, and deep color, which can result in a wine with low alcohol concentration, high acidity, and astringency. Li [9] and Lan [11] also reported that wines of native Chinese species had relatively higher blue % values and lower red % values.

4.1 *Vitis amurensis*

V. amurensis, which originated in north-eastern China, is now commercially cultivated in many places. The most important trait for this species is cold resistance. *Vitis amurensis* has a strong root system and high growth vigor, allowing it to survive at temperatures as low as −40°C. Besides, this species also showed high resistance to many diseases such as grape white rot and grape anthracnose [12]. Thus, it has been used as a disease-resistant stock as well as the most powerful cold-resistant rootstock to breed materials for resistance to biotic and abiotic environmental factors [12], and it is considered to be an effective way to save inputs in vineyard management by avoiding burying the vines.

Since the 1950s, significant progress has been made in understanding and utilizing wild *V. amurensis* grape germplasm resources in China. Grape researchers conducted a series of selection and domestication experiments on the *V. amurensis* species in Northeast China, and after many years of effort, they have selected a series of good varieties and types (**Figure 3**), as well as a series of work on cultivation and expansion on this variety [13].

As a wine grape, the *V. amurensis* fruit has a unique aroma and distinctive taste with high acidity and bitterness thus was used to make sweet wines [12, 14]. Nowadays, with the breeding of new varieties, *V. amurensis* and its hybrids can be used to make sparkling wine [15], rose wine [16], and ice wine [11]. Some novel techniques, such as carbonic maceration can also be used to improve the quality of *V. amurensis* wine [17].

When Bei Bing Hong (a variety of *V. amurensis*) was used to produced sparkling wine, its esters, carbonyls, alcohols, and terpenes contributed significantly to the aroma profile of the wine. The typical aroma characters of Bei Bing Hong sparkling wine are fruity aromas such as apple, apricot, pear, strawberry, cherry and sweet melon [15]. A mixed brewing method was used to produce rose wine from *Vitis amurensis* Rupr cv. Gongzhubai (white) and Beibinghong (red) grapes [16]. The

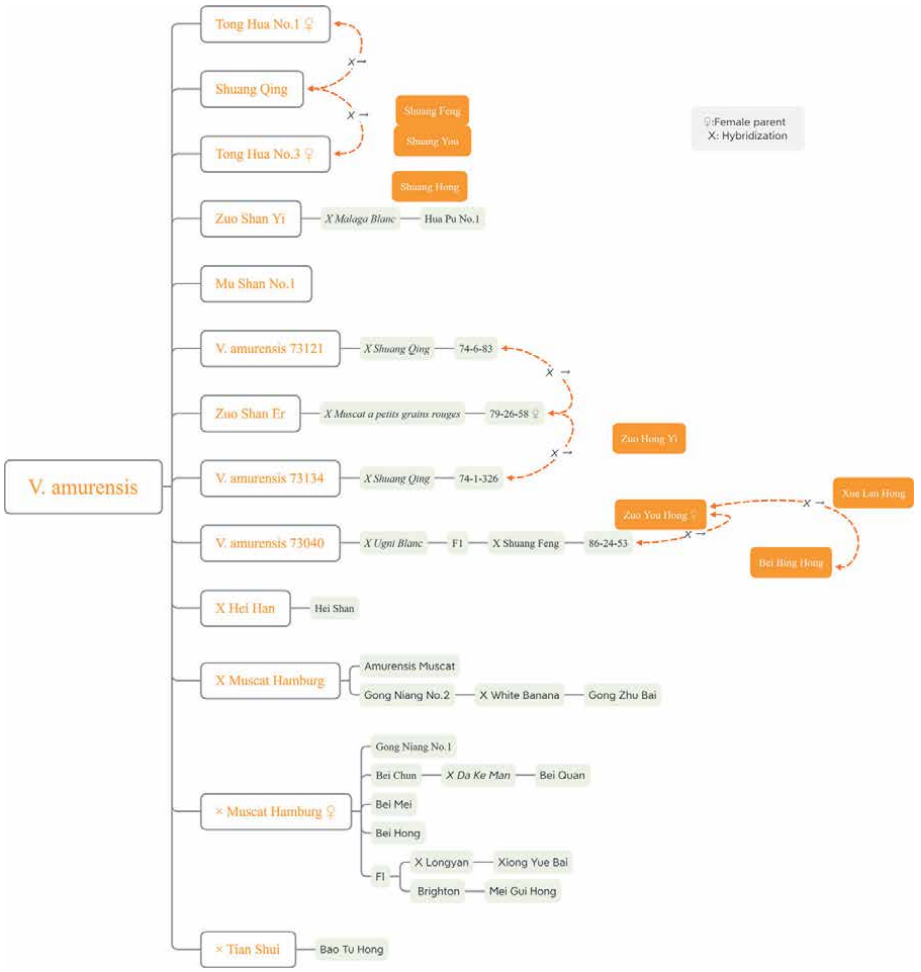

Figure 3.
Elite clones and hybrids varieties of V. amurensis.

fruit of each variety was pressed and the must fermented at low temperatures (11 ~ 12°C). By combining 8% and 12% of Beibinghong wine with Gongzhubai wine, a rose wine with elegance and aroma complexity was produced [16].

Lan [11] studied the evolution of free and glycosidically bound volatile compounds in 'Beibinghong' grape berries during on-vine, over-ripening, and freezing processes. The results showed that the aroma profiles of 'Beibinghong' icewine berries were characterized by C6 compounds, higher alcohols, and terpenoids in free fractions as well as carbonyl compounds, higher alcohols, C6 alcohols, and terpenoids in bound fractions. A striking alteration of the volatile profile of C6 alcohols, higher alcohols, and oxidative terpene derivatives occurred at sub-zero temperatures. These changes were attributed to a series of reactions (biotransformation, oxidation, and anaerobic metabolism) induced by water loss and particularly, freeze–thaw cycles [11].

Anthocyanins are responsible for the color of grapes and wine. Zhao [10] analyzed the anthocyanin profiles of grape berries of *Vitis amurensis*, its hybrids, and their wines. It was found that the anthocyanin profile of the grape cultivars consisted of 17 anthocyanins, including 11 anthocyanin monoglucosides and six anthocyanin diglucosides. However, the wines produced a slightly different result in anthocyanin distribution in the corresponding wines where 15 kinds of

anthocyanins, including six diglucosides and nine monoglucosides were detected [10]. Furthermore, pelargonidin-3,5-diglucosides was also found in the grapes and their corresponding wines.

Additionally, Li [9] also revealed that *Vitis amurensis* and its hybrids wines had a higher phenolic percentage of non-coumaroylated 3, 5-O-diglucosidic anthocyanins, while *V. vinifera* wines had a higher phenolic percentage of flavan-3-ols and 3-O-monoglucosidic anthocyanins.

4.2 *Vitis davidii* (spine grape)

Vitis davidii var. Forex belongs to the East Asian Vitis spp. and is one of the main wild grape species growing in the East Asian region. It is also known as Spine grape, because its shoots, petioles, and veins are densely covered by spines at 1–2 mm long [18]. The spine grape is mainly distributed in the mountains covered by the subtropical rainforest to the south of the Yangtze River. Huaihua county in Hunan province and Chongyi county in Jiangxi province are the most representative regions for spine grapes because of their wide distribution in those areas [19]. As spine grapes originated from the subtropical humid areas of southern China, this variety showed strong tolerances to high temperatures, high humidity, and resistance to diseases, such as spot anthracnose, white rot disease, and anthracnose [19].

Spine grape was used as table grape years ago, because of its larger berry size compared to other wild species, with an average fruit weight between 3.0–4.5 grams, and a total soluble solid range of 14.5%–16.0% [20]. Recently, with the rapid increase of cultivated area, only a small quantity of spine grapes was made available as fresh edible fruit and a major portion tend to be abandoned each year. Researchers have found that the intense process of converting the Spine grape to wine not only prevents the wastage of grape fruits but also brings high economic benefits to local growers [21]. More so, the development of new cultivars also promotes Spine wine production.

Meng analyzed the physicochemical parameters and aromatic components of nine clones of spine grape from Zhongfang County (Hunan Province, China) [22]. The berry weight, total soluble solids, titratable acids (expressed as equivalent of tartaric acid), and pH were found to be in the ranges of 2.08–3.88 g, 9.5–15.4 Brix, 1.99–3.93 g/L, and 3.16–3.77, respectively, indicating that the clones are more suitable for winemaking compared to the wild spine grape.

Flavor compounds are important quality indexes for wine production, which are mainly derived from grape berries, and can be affected by soil, altitude, slope, and cultivation management among others. In two different studies, Meng [22] and Zhao [18] respectively evaluated the free aromatic components and the influence of different altitudes on flavor compounds of Spine grape clones, 'Ziqiu', 'Seputao',' Miputao',' Xiangzhenzhu', 'Tianputao', and' Baiputao'. According to the findings, C6 compounds were the most abundant aromatic components in various spine grape clones, accounting for 71–94% of the total aromatic compounds identified. The most predominant compounds were (E,E)-2,4-hexadienal and (E)-2-hexenal [22]. At the height of 700 meters above sea level, the contents of anthocyanins, non-anthocyanin phenolic compounds, and aroma compounds in 'Seputao' were significantly higher than those at 240 meters and 600 meters altitudes. However, at the altitude of 240 meters, the contents of reducing sugars, anthocyanins, non-anthocyanin phenolic compounds, and aroma compounds in'Ziqiu'were the highest among three altitudes 240, 600, and 700 meters [18].

Meng [19] also investigated the phenolic profiles and antioxidant activity of four spine grapes cultivars (Junzi #1, Junzi #2, Liantang, and Baiyu) from Chongyi County, Jiangxi Province, China. It was revealed that Junzi #1 had the highest phenolic content and the strongest antioxidant capacity, HPLC analysis also showed

that the (+)-catechin was the most abundant phenolics while hydroxycinnamic acids were the major phenolic acids [19]. Regarding some individual phenolic compounds, JZ-1 contained the highest p-coumaric acid, coumarin, trans-resveratrol, and (+)-catechin contents, while BY had the highest rutin and quercetin contents.

The same researcher also characterized the phenolic profile of young wines made from spine grape. Like most vinifera wines, flavan-3-ols were the major class of phenolic compounds present in spine grape wines while quercetin-3-rhamnoside was the main singular flavonol [21]. In addition, syringetin-3-glucoside and dihydroquercetin-3-hexoside were the characteristic flavonols of red and white spine grape wines, respectively, while coutaric acid and fertaric acid were the dominant phenolic acids [21].

Organic acids play a key role in grape and wine quality. The acid component of grape berries mainly consists of tartaric acid, malic acid, lactic acid, acetic acid, citric acid, and oxalic acid. The total acidity in *Vitis davidii* Foex fruits is typically higher than in *Vitis Vinifera* varieties, resulting in high acidity in the fermented wine [23] (around 8 grams of tartaric acid per liter of wine after malolactic fermentation), which has been a major constraint on the Spine wine industry.

The effect of deacidification reagents ($KHCO_3$ and $CaCO_3$) on the aroma compounds of spine wine was studied by Li [23]. The results showed that the OAVs of compounds with flavors of fruit, cheese, caramel, and chemical were reduced. However, sensory evaluation revealed that the mouthfeel and aroma characteristics of spine wine were improved after deacidification.

Due to the relatively low sugar content in Spine grapes, ranging from 12.3 to 15.9°Brix, an early winemaking study showed that sugar addition was required for red Spine wine production to improve wine quality [24]. Conversely, this neutral grape characterized by low sugar levels and high acidity is suitable for making distilled spirit-based beverages [25].

Currently, high quality Spine grape spirits are produced by several local wineries and are welcomed by local consumers. Xiang [26] identified the key odor-active volatile compounds in the head, heart, and tail fractions of freshly distilled spirits from Spine grape (*Vitis davidii* Foex) wine. The volatile compounds had considerably varying amounts in the head, heart, and tail fractions due to differences in boiling point and solubility, which resulted in various evolution patterns during distillation. The head fraction was characterized by fruity, fusel/solvent notes owing to higher concentrations of higher alcohols and esters, while the tail fraction had more intense smoky/animal, and sweaty/fatty attributes due to higher concentrations of volatile phenols and fatty acids [26].

4.3 *Vitis quinquangularis* Rehd

Vitis quinquangularis, known locally as the pentagon-leafed grape, is distributed south of the Yellow River in regions that have sufficient sunshine and are at an altitude of <1500 m.

Vitis quinquangularis is an important research grape with high resistance to powdery mildew due to its high resveratrol content [27].

Selection studies have also been conducted on *V. quinquangularis* in the central part of China. Liang [28] revealed that this cultivar contained different anthocyanins compared to *Vitis davidii*. For example the 'Xiangshan No. 4' (*V. quinquangularis*) contains high levels of 3′,4′-substituted anthocyanins, low levels of flavonols, and low 3′,4′-substituted flavan-3-ols, indicating that the F3′H branch pathway is the principal carbon pathway synthesizing mainly 3′,4′-substituted anthocyanins [28].

Also, the grape berries of *Vitis quinquangularis* ripen with low sugar content and high acidity, but with dark-colored skin. Their wines have a characteristic varietal aroma and a pronounced acid and tannic sensation [28, 29].

Fang examined the effects of different processes on the flavor components of wild *V. quinquangularis* wine produced in the Qinba mountain region [30]. The findings demonstrated that alcohol was the most important aroma compound in *V. quinquangularis* wine, with the highest relative contents of benzene ethanol and pentanol. After six months of aging, the aroma quality of carbonic macerated wine was better than that of the traditional process [30].

Liu also proved that carbonic maceration increased the contents of esters, acids, and phenols as well as the species and contents of volatile compounds in wines [31]. The combination of carbonic maceration and malolactic fermentation could result in more volatile compounds in wines, giving such wines a unique taste distinct from traditional wines [31]. Similar results were reported in *V. amurensis* wines, with Pei revealing that carbonic maceration decreased the fruit aroma while increasing the flower aroma and overall aroma quality of *V. amurensis* wine [17].

5. Buried viticulture

In China, most of the viticulture regions are distributed in cold and mid-temperate regions (**Table 2**), these regions are typically affected by the continental monsoon climate with cold, dry winters, and frequent early spring frosts, which can result in severe freezing injury and dehydration risks to branches and roots [32, 33]. It has been acknowledged that, as the main cultivated wine grape variety, the grape and wine quality of *Vitis vinifera* is higher than that of *Vitis labrusca* and various wild species, however, the cold resistance is completely opposite [34]. When the temperature in winter is extremely lower than −15°C, the vines need to be protected to withstand the severe cold, prevent draining, and ensure its safe overwintering. In China, more than 90% of *Vitis vinifera* are distributed in areas where the vines must be buried under a layer of soil during winter (buried viticulture).

In order to choose suitable measures for overwintering, interspecific hybrid breeding, rootstock grafting, wind dispersing cold air, adjusting plant load, soil or material covering, delaying pruning, and other technics were implemented by numerous of researchers all over the world [34, 35]. However, after years of experiments, burying the vines into the soil is still the most effective way to protect vines over winter. In general, the vines are taken down off the trellis after pruning and then buried into the soil (more than 30 cm underground) in the winter, and the soil is removed before the sprouting in the next spring. Both artificial and mechanical methods are used to complete the burying and unearthing of the vines, and this work should be done very carefully to prevent damage to branches and buds. To aid buried viticulture, several cover materials and methods, such as film mulching, industrial cotton, straw mattress, and plastic have been devised and used. Additionally, various types of vine burying and soil removing equipment (or digging machines) have been designed and employed [36].

Because buried management exposes the soil surface in winter and early spring, there is an increased danger of wind erosion and sandstorms, which may cause ecological problems in viticulture regions in northern China. Recently, a new viticultural procedure was reported during winter pruning to ameliorate this phenomenon, by clutching the vine shoots on the wires until next spring. Also, a windbreak was built as a protective function to reduce wind speed, and the dangers of sand storms as well [37].

In conclusion, buried viticulture is labor intensive, costly, and has the potential to cause damage and diseases to branches while also destroying the ecological environment. Buried viticulture further limits mechanized production and all these challenges are serious impediments to China's wine development [34].

6. Winemaking techniques

Nowadays, with a decrease in wine consumption and an increase in imported wines, there is no mention of competition from Chinese liquor -Baijiu, Chinese rice

Technics	Treatment	Mechanism	Major impacts on wine composition	Reference
Berry heterogeneity	Berry classification	Heterogeneity influence fruits weight, diameter, berry density, and soluble solids content	Smaller fruits reduced the contents of malic acid and pH value, increased wine color, phenolic substances, varied the aroma substances and titratable acids contents	[38]
Cold maceration	Temperature below 10°C for 3-7 days	Lower temperature improved the maceration time and substance from grape skins	Improving wine color and aroma	[39]
Carbonic Maceration	Sealed tank with CO_2 at 30–35°C for 8–15 days	Anaerobic metabolism by berry enzymes	Reducing acid, color, and tannin, improving aroma quality	[40]
Flash evaporation	Heat must to 85–91°C by steam at −0.9 Pa	Break down the skins at high temperature with decompression condition	Increasing the extraction of total phenols, anthocyanidin, and aroma compounds	[41]
Saignée	30% of juice was released after 12 hours	Removing juice to increase skin ratio of red wine	Simultaneous production of dry-red and rose wines, increase the color, aroma intensity, and antioxidant properties of red wine	[42]
Pulsed electric field	3000 Hz, 10 pulse, with 6.5-35kv/cm electric field intensity	Electrical breakdown, electroporation perforated theory	Increasing phenolic profile and wine color	[43]
High hydrostatic pressure	Grapes were subjected to HHP treatments (200-550Mpa) for 10 min	Provide the activation energy for extraction chemical compounds at low temperature without break covalent bonds	Controlled microbial populations, increased phenolic compounds, and anthocyanin extraction, returned higher aromatic quality and color scores in wine	[44]
Withering	Loss of water by 20–40%	Concentrated the grape substance by dehydration	Increased alcohol, residual sugar, and acidity content, improved, phenols, antioxidant activity, brightness, yellow tone, aroma, and taste	[45]

Table 3.
Pretreatment techniques before fermentation.

wine, and beer, and domestic wine production in China has decreased year by year since 2012. It is now a common phenomenon in the global wine industry where total wine production exceeds demand and as such, China's wine manufacturers will continue to face great pressure in the coming years. To preserve the wine market, enologists and researchers must improve wine quality, increase shelf life, and produce new products.

In this chapter, some useful pretreatment techniques, such as berry heterogeneity, cold maceration, carbonic maceration, flash evaporation, saignée, pulsed electric field, high hydrostatic pressure, and withering procedure are further reviewed (**Table 3**).

7. Conclusions

China has become one of the most important wine countries in the world, the history and current situation of Chinese grape and wine industry were reported. According to the meteorological and geographical regionalization, China wine producing area have been categorized into 11 regions, the detailed information of these regions was listed.

In many parts of China, Vitis wild species such as *Vitis amurensis*, *Vitis davidii*, and *Vitis quinquangularis* and their hybrids varieties were wildly planted and used as resistant stock, however, the elaborated wine made from these grapes were quite unlike those made from *Vitis vinifera*, thus, chemical components and wine making technics of wild species were summarized. Finally, the impacts of some pretreatment techniques on *Vitis vinifera* wine composition and quality were reviewed.

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Author details

Ma Tengzhen[1,2*] and Han Shunyu[1,2,3]

1 College of Food Science and Engineering, Gansu Agricultural University, Lanzhou, China

2 Gansu Key Laboratory of Viticulture and Enology, Lanzhou, China

3 China Wine Industry Technology Institute, Yinchuan, China

*Address all correspondence to: matengzhen@hotmail.com

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Chapter 15

White Wine Protein Instability: Origin, Preventive and Removal Strategies

Luís Filipe-Ribeiro, Fernanda Cosme and Fernando M. Nunes

Abstract

White wine protein instability depends on several factors, where *Vitis vinifera* pathogenesis-related proteins (PRPs), namely chitinases and thaumatin-like proteins, present an important role. These proteins can be gradually denatured and aggregate during wine storage, developing a light-dispersing haze. At present, the most efficient process for avoiding this wine instability is through the removal of these unstable proteins from the wine before bottling. To remove unstable white wines proteins, the sodium bentonite fining is the most used treatment, however, many alternative techniques such as ultrafiltration, the application of proteolytic enzymes, flash pasteurisation, other adsorbents (silica gel, hydroxyapatite and alumina), zirconium oxide, natural zeolites, chitin and chitosan, carrageenan and the application of mannoproteins have been studied. This chapter overviews the factors that influenced the white wine protein instability and explored alternative treatments to bentonite to remove white wine unstable proteins.

Keywords: white wine, protein instability, thaumatin's and chitinases, treatments

1. Introduction

Even wine contains a low level of proteins and glycoproteins, which usually ranged from 15 to 230 mg/L, proteins present an important role from a technological point of view [1]. Indeed, proteins greatly affect wine quality by contributing to its sensory and foam characteristics [2–4]. However, specific wine proteins, mainly grape wine unstable proteins, can be gradually denatured and aggregate/precipitate during wine storage, developing a light-dispersing haze, this being the main cause of post-bottling haze development in white wines [5]. Even the formation of protein haze is improbable to affect the olfactory or gustatory wine characteristics, turbid wines are usually rejected by consumers, causing significant economic losses for the wine industry and brand image [6–8]. Wine proteins are mainly derived from grapes, but they could also be formed by the metabolism of the several microorganisms (yeasts and lactic acid bacteria) present in the vinification process [9]. Most of these proteins are extinct after wine alcoholic fermentation and consequent fining processes. Nevertheless, the so-called pathogen-related (PR) proteins (β-glucanases, chitinases, thaumatin-related proteins) can persist in the final wine as they are resistant to proteolysis and low pH [10]. They are produced by the grapevine for defence against bacterial or fungal infections and in reaction to abiotic stress [11]. These proteins are resistant to the wine acid conditions, heat and proteolysis due to

their compact structures [9]. Numerous research works have stated that wine total protein levels could not predict wine protein instability, as individual protein fractions synthesised in grape berries are responsible for haze development [12, 13]. Furthermore, the wine chemical composition such as metal ions, ionic strength, pH, alcohol level, polysaccharides and phenolic concentration could also play an essential role in protein haze development as these parameters might affect protein denaturation [8, 14]. Additionally, Pasquier et al. [15] mentioned that the climatic changes, with the rise in temperatures and the reduction in precipitation throughout the grape maturation phase, lead towards increase in the probability of wine protein instability. In the winemaking industry to avoid this instability, normally the unstable proteins are removed through bentonite fining before wine bottling. However, this fining agent is non-specific, removing other wine compounds besides unstable proteins, which may affect wine sensory qualities [16]. Therefore, in the previous years, several alternative solutions to bentonite fining for this aim have been searched [16, 17].

2. White wine protein instability

2.1 Proteins responsible for white wine protein instability

Several studies were performed concerning wine protein instability. Koch and Sajak [18] verified using electrophoresis that the heat-formed deposits enclosed two types of protein fractions with diverse heat sensitivities. Moretti and Berg [19] after fractionation and analysis of wine proteins concluded that, among grape and wine proteins, that the protein fractions with low isoelectric points and low molecular weights were more sensitive to heat treatment and responsible for wine protein instability. The main proteins related to the white wine protein instability have a low molecular weight (12.6–30 kDa) and isoelectric point (4.1–5.8) and contain glycoproteins [20]. Waters et al. [21] did the separation and the fractionation of wine proteins using a combination of salting out with ammonium sulphate and ultrafiltration, showing that the protein fractions with those characteristics (24 and 32 kDa) were more sensitive to high temperatures, contributing more to the white wine protein instability. The lower-molecular-weight protein fractions appear to be the major responsible for white wine haze, where the protein with 24 kDa produced nearly 50% more haze with identical concentration than protein fractions with 32 kDa [13]. Many works showed that pathogenesis-related proteins (PR) are the principal responsible for wine protein instability [13, 22]. Pathogenesis-related proteins are very important for plant protection and are associated with its disease resistance, growth and adaptation to stressful environments [23]. *Vitis vinifera* is the most used for winemaking; however, it is very sensitive to pathogens, particularly fungi and oomycetes, such as *Botrytis cinerea* and *Plasmopara viticola*, respectively [24]. Pathogenesis-related proteins (PR) are produced by the plant in response to infection by pathogens [25], to control the harm made to the grapevine [26]. In *V. vinifera* grape varieties, the thaumatin-like (PR-5 type, 24 kDa protein fraction) [13] and chitinases (PR-3 type, 28 kDa protein fraction) [13] are the two main pathogenesis-related (PR) proteins separated from wine, presented a globular structure and at wine pH a positive charge [27]. Other examples of PR proteins existing in lesser quantity in wine are osmotins, β-1,3-glucanases, invertases, lipid transfer proteins [28]; however, diverse isoforms of thaumatin-like proteins and chitinases have been recognised in grape musts of several *V. vinifera* grape varieties, with a molecular weight between 20 and 30 kDa, and an isoelectric point between 3.0 and 5.0 [7, 29]. These are the principal soluble proteins from *V. vinifera* [22, 30] and are responsible for haze development in bottled white wine during storage and transportation [27, 31]. These proteins are synthesised

during grape development in function of the grape variety [32], region and year [24, 28] presenting higher levels in the ripening; that means that riper grapes are the more susceptive to protein instability [30]. Chitinases and thaumatin-like proteins present a significant amount of disulphide bonds that contribute to their chemical stable structures and some resistance during the vinification process (some resistance at the low grape juice pH (3.0–3.8)) and resistance to proteolysis [5, 33]. However, the low-molecular-weight proteins (chitinases) are sensitive to temperature changes [22] and wine pH [34]. Thaumatin-like proteins are more thermostable and insensible to wine pH variations, showing no significant structural variations or aggregation in different wine pH [34]. The different sensibility of chitinases and thaumatin-like proteins appears to be associated with the differences in the secondary structure of both proteins, elliptical for chitinases and globular for thaumatin-like proteins [34, 35]. The pathogenesis-related proteins are present in different concentrations in the grape juice of the diverse grape varieties of Sultana, Sauvignon Blanc, Pinot Noir, Muscat of Alexandria and Shiraz with chitinases/thaumatin-like proteins of 118/119, 76/119, 44/23, 21/35 and 9/18, respectively [30]. These researchers likewise verified that grape berry destruction throughout mechanical grape picking, related to long-distance transportation, could encourage the production of pathogenesis-related proteins by the grape defence mechanism before grape pressing [36]. In fact, chitinases and thaumatin-like proteins and its variances in heat stability give the impression that protein composition may influence haze development in wines [9, 37]. Thaumatin-like protein (24 kDa fraction) is the principal responsible for haze formation relative to chitinase (32 kDa fraction) [21, 38]. However, chitinases are very sensitive to precipitation, where a high correlation was verified between wine chitinases levels and wine haze obtained [37]. The thaumatin-like proteins shows a melting temperature of 62°C, with a determined denaturation half-life of 300 years at 25°C, and chitinases present a denaturation half-life of 6 minutes at 55°C, consequently extrapolating down to a denaturation half-life of 3 days at 35°C or 2 years at 25°C. It was observed that vacuolar invertase (GIN1), from the grapes, and β-(1–3)-glucanases (32 kDa fraction) can also influence haze formation. In fact, the existence of *V. vinifera* thaumatin-like protein bands, β-(1,3)-glucanase and maturation-related protein-like (27.4 kDa) Grip22 precursor have been associated with the natural protein haze of white wines [13, 39, 40]. In fact, there is not a correlation between the total amount of protein and wine protein instability [39].

2.2 Factors that can affect white wine protein stability

The denaturation of some white wine proteins could result in aggregation and flocculation and sometimes in the development of deposits [25], other wine non-proteinaceous compounds can also be related to the wine protein haze development. Curiously, wines with an identical protein fraction can present different haze tendencies [41], and the wine ethanol level did not influence wine protein instability [42, 43]. The protein-polyphenol interaction is the major studied mechanism associated with white wine protein instability [44, 45]. The existence of procyanidins is necessary to develop wine turbidity, only the presence of wine proteins did not develop wine turbidity [46]. It was shown that the interaction between haze-active polyphenol and haze-active protein and the amount of haze formed is highly dependent on protein and polyphenol concentration and their ratio [47]. The turbidity of a protein-polyphenol complex increased with a pH rise from 2.5 to 3.7 (model wine solution with 10% ethanol) [48]. Some authors consider that protein haze formation is an isoelectric precipitation mechanism [49]. Some authors think that turbidity formed in white wines is related to hydrophobic interactions among proteins and tannins happening on the hydrophobic tannin-binding sites of proteins that can be

exposed depending on heating and reduction [37]. Many phenolic compounds were detected in protein haze, such as tyrosol, *trans-p*-coumaric, vanillic, *trans*-caffeic, protocatechuic, gallic, syringic, ferulic, shikimic acids, (+)-catechin and ethyl coumaric acid ester; quercetin and cyanidin, after acid hydrolysis, the existence of procyanidins was also shown [39]. Phenolic compounds can increase haze formation by cross-linking denatured proteins provoking aggregate development [9], in fact, the removal of phenolic compounds from wines resulted in reducing haze development [38]. The X factors are factors essential for protein turbidity and are wine conditions such as pH, ionic strength, organic acid concentration [49], polysaccharides [50], metal ions [51] polyphenols/phenolic compounds [25] and sulphate anions. As mentioned before, wine pH is an important factor in protein haze development, with model wines at pH 4.0 inducing higher protein aggregation and turbidity development after heating than model wines of lower pH (pH 3.0) [52]. The application of sulphate anions or sodium cations that increase the wine electrical conductivity and ionic strength increases the tendency of haze formation after heating, by the decrease of the electrostatic repulsion of proteins [37]. In model wines, it was shown that other ions including tartrate, chloride, $Fe^{2+/3+}$ and $Cu^{+/2+}$, do not influence the turbidity formation [30]. Higher electrical conductivity (0.134 and 0.163 S/m) and protein levels (9 and 25 mg/L) provoke greater perceptible turbidity; however, the white wine with low iron levels (0.3 and 0.9 mg/L) and protein stability appears to increase so there is a negative correlation between wine turbidity and the iron levels [53]. There is evidence that polysaccharides could potentially decrease wine protein instability by forming a protective layer around unfolded proteins [54]. Organic acids could present interactions with phenolic acids, free amino acids, tannins, pectic compounds and sulphate ions, avoiding in this manner, their interaction with proteins [55]. The same authors verified that organic acids could influence wine protein instability by the electrostatic interactions that depend on the organic acid pKa and protein isoelectric point values and the medium pH. The sulphate ions could be a non-proteinaceous factor for protein instability, as they promote protein-protein hydrophobic interactions Pocock et al. [38], in addition to the suppression of the electrostatic repulsion between proteins by the increase of the ionic strength of the medium [37]. It was demonstrated that potassium hydrogen sulphate can influence haze formation [7]. Some authors suggest a three-stage process in the protein haze formation that included protein unfolding, protein self-aggregation and aggregate cross-linking, highlighting the role of sulphate ions in all stages [9]. Chagas et al. [56] verified the influence of sulphur dioxide, existing in wines in the irreversible denaturation and aggregation phenomena of thaumatin-like proteins and their influence on wine protein instability or turbidity development. The presence of ion bisulphite (HSO_3^-) results in cleavage of the disulphide bonds of the thaumatin-like proteins, with the formation of S-thiosulfanates and free thiol-groups that contribute to the temperature-induced protein unfolding. The hydrophobic surfaces and the presence of free thiol-groups result in protein aggregation by formation of inter-protein disulphide bonds in thaumatin-like proteins, following a nucleation-growth kinetic mode.

3. Preventive treatments and strategies to mitigate white wine unstable proteins

3.1 Effect of growing and harvest conditions on wine protein composition

By using principal component analysis and clustering techniques, Sarmento et al. [32] pointed out that the most important factor affecting wine protein profile was the grape variety, and the growing region, whereas vinification practice

(industrial and laboratory scale) on the same varietal wine did not show a major effect. In grapevines, the synthesis of the PR proteins is regulated in a developmental and tissue-specific manner and occurs predominantly in the skins of the grapes [36, 57]. In *V. vinifera* cv. Muscat Gordo Blanco, both the concentration of the corresponding main thaumatin-like proteins and the berry-specific expression of the VvTL1 gene improved intensely after *véraison* and continued during grape maturation [58]. Identical developmental patterns were also found in the expression of genes encoding chitinases, some identical to those involved in wine protein haze [59–61]. Immunological research of *V. labruscana* cv. Concord likewise demonstrated that thaumatin-like proteins and chitinases accumulate during berry maturation [62]. PR proteins also exist in several other fruits such as banana [63], cherry [64] and kiwi fruit [58]. In all *V. vinifera* cultivars studied, thaumatin-like proteins and chitinases are the main soluble protein of grape berries [36, 58]. The prevalence of these PR proteins was evident at all phases of the grape berry growth next *véraison* [30]. Significantly, as the levels of extractable proteins in the grape berries continually rise during maturation, it can be supposed that the haze-forming potential growth as maturation continues [30, 58]. Pocock et al. [30] also showed that the increase of thaumatin-like proteins and chitinases initiated at berry softening for Muscat of Alexandria, Sultana, Shiraz grape varieties, Sauvignon Blanc and Pinot Noir grape varieties. As in healthy grape berries, PR protein synthesis seems to be caused by *véraison*, this does not signify that the traditional PR protein inducers, wounding, stress and pathogenic attack, cannot additionally modulate the grape berries' PR proteins concentration. These grape proteins in vitro display antifungal activity to *Botrytis cinerea*, *Uncinula necator*, *Phomopsis viticola*, *Elsinoe ampelina* and *Trichoderma harzianum* general fungal pathogens of grapevines [35, 62, 65–67]. The antifungal activity shown in vitro replicates the major function of the PR proteins *in vivo*, their expression in grapes afterward *veraison* represents a defence mechanism for grapes. Jayasankar et al. [66] give additional credibility to this hypothesis by indicating that after *in vitro* selection, grapevines regenerate with *E. ampelina* culture filtrates presented high constitutive expression of PR proteins, comprising VvTL1 and higher disease resistance. Works in which the PR proteins synthesis is changed by gene technology would permit us to explore this hypothesis more. Currently, there are slight chances that the wine turbidity problems could be resolved by decreasing the PR protein expression in grape berries as this could lead to the grapevine disease. In leaves and grape berries from infected grapevines with pathogens, improved expression of some PR genes and higher levels of some PR proteins have been shown [68–70]. In greenhouse experimentations, Monteiro et al. [67] showed in infected grape berries with *U. necator* augmented concentration of thaumatin-like proteins than in uninfected grape. Jacobs et al. [68] observed that in response to powdery mildew infection β-1,3-glucanase activity and chitinases augmented in leaves and grape berries, and that genes expression (VvGlub, VvChi3 and VvTL2), for coding PR proteins, was powerfully induced. Only VvTL2 of the three putative gene products has been found as a soluble protein in grape must and wines [13]. In Chardonnay *V. vinifera* cv. grape bunches, Girbau et al. [71] showed that occasioned powdery mildew infection augmented the concentration of a grape berry lesser thaumatin-like proteins, VvTL2, in wine. In infections with higher intensities (>30% of infected bunches), the wine turbidity values measured after a heat test were significantly higher. Marchal et al. [72] showed that grape must from infected grape berries by *B. cinerea* presented lower protein concentration, in opposing to expectations that fungal diseases would lead to higher concentration of PR proteins in grape, and suggested that proteolytic enzymes from *B. cinerea* were responsible for this. In culture media and on fruits such as apple, secretion of proteases by

B. cinerea has been observed [73] and in tomato [74]. Girbau et al. [71] also studied the influence of *B. cinerea* infected grapes on the vineyard and observed that infection resulted in noticeable reductions in the concentration of PR proteins in the grape berries. Similar although fewer tendencies of decreases in protein concentration were observed in laboratory experimentations in which otherwise healthy grape berries were inoculated with *B. cinerea* [71]. In this work even though these grapes were not vinified, the variance in protein concentration was predictable that between uninfected and infected grapes would also be shown in wines produced from uninfected and infected grapes. In the grape juice from *Botrytis*-infected grape berries, the decrease in protein concentration did not appear to be an artefact of reduced extraction into juice due to desiccation or shrivelling of the fruits, nevertheless could be due to proteolytic degradation of grape PR proteins by enzymes of *B. cinerea* as suggested by Marchal et al. [72]. In grape must, protein concentrations were also decreased when in this medium *B. cinerea* was grown [71]. If these effects are due to the activity of proteolytic enzymes from *B. cinerea*, these enzymes have the capacity to substitute bentonite fining for protein stabilisation in oenology, an objective of many research efforts worldwide. The consequences of mechanical grape picking, a harvesting operation that could cause wounding, on the grape berries PR proteins concentration, are therefore of attention. Paetzold et al. [12] showed that grapes picked up by hand, originated grape must with lower protein concentration compared with that of mechanically harvested grapes. The absence of stalk throughout crushing led to lesser polyphenolic concentration in the grape juice compared with the grape juice from grapes picked up by hand, therefore fewer proteins were lost in complexes with phenolic compounds from grape juice from the fruit picked up mechanically. Dubourdieu and Canal-Llaubères [75] showed that wine produced with destalked grapes with maceration during 18 hours presented higher protein concentration than wine produced immediately by pressing of whole bunches. It was not elucidated, if this rise in protein concentration was due to the wounding of grapes that occurs during destalking or maceration or from the elimination of the grape stalks. Pocock and colleagues [36, 76] observed the influence of mechanical harvesting on the PR proteins in grapes and wine. Mechanical harvesting together with long transport of the grape berries leads to greater PR protein concentration in the grape juice and wine. Indeed, white grapes harvested mechanically, following transport was found to double the concentration of bentonite necessary for the avoidance of protein haze when compared with grape berries harvested by hand and transported from the same vineyard [76]. This does not seem to be a consequence of an increase in protein synthesis, as evaluations among hand picked up grapes, mechanical picked up intact grapes, and the major form of mechanical picked up grape berries—a combination of damaged grape berries and grape must—showed that few if any protein was formed as a consequence of stress provoked by mechanical grapes picked up. Protein concentration increase in grape must from mechanical picked up grape berries consequently look to be due to protein extraction from grape skins rather than a physiological wounding answer by the grapes. The influence of water stress established under some viticultural management practices has been studied, on the PR proteins expression in grape berries by determination of the PR protein concentration of *V. vinifera* cv. Shiraz grape berries in irrigation essays [30]. The absence of irrigation, did not lead to higher PR proteins concentration in the grape, however it provided a clear physiological marks of grapevine water stress. On a fixed quantity of protein per grape, it was observed that in the grape must from water stressed grape the protein content was greater than that from irrigated grape since grapes from irrigated grapevines were greater and thus grapes solutes were

fewer concentrated. The water stress influence on the grape dimension is an overall phenomenon [77] and it is probable that reports related to the wine turbidity problems, are higher in drought years and they are due many to a variation in the grape dimensions in these years instead of a direct physiological answer of the grapes to water stress in the formation of PR protein.

3.2 Preventive winemaking practices to avoid white wine protein instability

Regarding the mechanisms of wine protein turbidity development, there are numerous potential approaches for avoiding wine turbidity that would either decrease or remove the requirement for bentonite application. These comprise reducing the concentration of wine phenolic compounds; reducing the wine ionic strength; disrupting hydrophobic protein-protein interactions; stabilising wine proteins against thermal unfolding; degrading wine proteins enzymatically after heat treatment; application of alternative adsorbents or ultrafiltration to eliminate proteins [9].

Enzymes application to degrading haze-forming proteins in wine is a specially an attractive substitute to bentonite since it diminishes aroma removal and wine losses. Preferably, active enzymes would be applied to grape must without the requirement for future removals, as in the case of glucanases and pectinases [78]. The products of grape proteins degradation may also be used by yeast as nitrogen sources, theoretically decreasing the common necessity for nitrogen application and enhancing wine aroma quality [79, 80]. For wine protein degradation, there are two important kinds of enzymatic activities: the decrease of disulphide bonds by protein disulphide reductases and the hydrolysis of peptide bonds by proteases [81]. The difficulty in using proteases for specifically degrading haze-forming proteins in wine is related to the stability of the proteins in wine-like conditions. Protein disulphide reductases could, hypothetically, destabilise and precipitate haze-forming proteins throughout vinification via reduction of disulphide bonds [22]. Nevertheless, under wine conditions, there have been no published cases of active protein disulphide reductases.

In a Champenois Chardonnay wine, it has been shown that a 24/25 kDa protein was an N-glycosylated protein and underwent no modification throughout fermentation [82], whereas degradation or variation of the sugar moieties of the glycoproteins (12–30 kDa) was found to happen during winemaking for a hybrid grape variety (Muscat Bailey A) [83]. The hydrolysis of the sugar chains of grape derived glycoproteins by glycosidase treatment was found to rise turbidity with seed phenols in a model wine [84]. Instead, yeast-derived mannoproteins (420 and 31.8 kDa) could contribute to a stabilisation effect on wine proteins, decreasing haze development [85, 86]. Yeast derived mannoproteins (10–30 kDa) possessing both compositions of the hydrophobic and hydrophilic protein domains and mannose moiety also improved the foaming properties in sparkling wines [87, 88].

Another strategy to decrease the level of proteins in white wines is pre-fermentative skin maceration, for example, in the Albariño grape variety, pre-fermentative skin maceration augmented the concentration of polysaccharides and phenolic compounds extracted, however, reduce the quantity of protein extracted, mainly of the pathogenesis-related proteins, specifically the *V. vinifera* chitinases and thaumatin-like proteins. While the PRPs and total protein of the Albariño wine produced by pre-fermentative skin maceration were lesser, the wine presented higher protein instability in the heat test, perhaps the presence of higher level of polyphenols compounds [17].

4. White wine unstable proteins removal

4.1 Physical treatments

The removal of unstable white wine proteins could be performed by the use of ultrafiltration [5, 20, 87–90], flash pasteurisation [91–93], high hydrostatic pressure [10] and ultrasound [94].

Hsu et al. [20] ultrafiltered a white Gewürztraminer and Riesling wine with Romicon and Millipore systems, worked with membranes of nominal molecular weight cut-offs (MWCO) of 10–100 kDa. According to these authors, protein stability could be achieved with MWCO of 10 and 30 kDa; nevertheless, if the protein stability was not achieved, bentonite required was reduced from 80 to 95%. However, according to Miller et al. [95] and Flores et al. [87, 88, 96], ultrafiltration could also lead to the depletion of wine aroma compounds responsible for the floral, fruity and honey/caramel descriptors (**Table 1**), changing, in this manner, the wine aromatic profile [97, 98]. Additionally, wines treated by ultrafiltration also showed a significant reduction in yellow colour (420 nm) and total phenols [87, 88], as well as a decrease in the 'body' and 'mouthfeel' related to the removal of colloids [99]. Furthermore, the high operation and equipment cost associated with the aroma decrease, making this procedure unattractive to the wine industry for eliminating unstable proteins.

Wines heat treatments at medium temperature (45°C, several hours) and high temperature (90°C, 1 minute), with and without the application of proteolytic enzymes, lead to a decrease of the wine protein level and up to 70% of the bentonite needed for heat stability [91]. However, after sensory assessment of the wines submitted to the different treatments the panel members in some wines submitted to heat treatment without enzyme application and to heat treatment with enzyme application (Trenolin blank, 10 mL/L), observed slight effects on wine aroma descriptors [91].

The results obtained by Tabilo-Munizaga et al. [10] established that high-pressure treatments changed the β-sheet and α-helical structures of wine proteins. During 60 days' storage period, the α-helix structure in high-pressure treatment samples was reduced. Structural modifications by high-pressure treatments (450 MPa for 3 and 5 minutes) increase wine proteins thermal stability and consequently delay the wine haze formation throughout wine storage.

	White Riesling			White Gewürztraminer	
Descriptors	**Control**	**UF1**	**UF2**	**Control**	**UF**
Overall intensity	5.97	5.33	5.23		
Fruity	4.83	3.93	4.10	5.22	4.39
Fresh fruit citrus	3.47	2.87	2.43	4.61	3.39
Floral	3.63	1.83	2.00		
Vegetative	1.93	2.63	3.13		
Cooked vegetative				1.97	2.22
Honey/caramel	2.43	1.63	1.57		
Chemical	4.07	3.97	4.33	2.35	3.56

[*] Scored on a nine-point intensity scale (1 = none, to 9 = extreme); UF, ultrafiltration.

Table 1.
Mean scores[*] of the significant aroma descriptor ratings for white Riesling and white Gewürztraminer wine (adapted from [97]).

Recently, Celotti et al. [94] developed a research work focused on the application of ultrasound for white wine protein stabilisation. The results showed that higher amplitude (90%) and treatment time (10 minutes) induced an increase in white wine protein stability. This effect is related to the protein charge neutralisation and surface electrical charges, intending positive conformational modifications in the wine proteins. This technique could be considered as a way to prevent wine protein precipitation and to decrease the amount of bentonite fining agents used in wineries.

4.2 Enzymatic treatments

Proteases hydrolyse the peptide linkages between the amino acid units of proteins. Protease activity exists in grape berries [100, 101] and yeast [101–108] as described by several authors. One important aspect is their potential role in wine protein haze reduction [90, 109]; however, proteases have low activity concerning haze-forming proteins, which consequently persist during the vinification process. It is essential, that proteases have to be active under specific wine conditions, namely acid pH, the existence of ethanol, sulphites, phenolics and if possible act at low temperatures. One more challenge is the resistance of PR proteins against proteolysis due to their molecular features such as disulphide bonds and glycosylations. However, proteases from plants (papain from papaya, bromelain from pineapple) have been tested with some promising results concerning their effectiveness in the degradation of heat-unstable proteins from white wine [110–113]. However, the search for fungal enzymes that could degrade wine proteins has so far remained ineffective [114]. As unfolded proteins are more easily cleaved by enzymes, the subsequent phase was the evaluation of the mutual effects of protease addition and heat treatment. Heat treatment joint with the application of proteolytic enzyme can decrease the formation of white wine protein instability; however, the low specificity of commercially disposable proteases for the haze-forming proteins seems to decrease significantly the possibilities of offering this strategy as shown by Pocock et al. [91]. A fungal acid protease resulting from *Aspergillus* sp. rich in aspergillopepsin I (EC 3.4.23.18) and aspergillopepsin II (or aspergilloglutamic peptidase, EC 3.4.23.19) in association with flash pasteurisation (75°C) of the grape juice was confirmed to eliminate haze-forming proteins and consequently stabilises the wines [92, 93]. The application of aspergillopepsin I to eliminate haze-forming proteins in grape must and wine is already authorised by the International Organisation of Vine and Wine [115] Resolution OIV-OENO 541A-2021 and Resolution OIV-OENO 541B-2021. Aspergillopepsin is active at juice and wine pH and at a temperature greater than the melt temperature of haze-forming proteins (chitinases and TLPs, 56 and 62°C, respectively). Therefore, after application of aspergillopepsin I, one short-term heating (60 and 75°C; 1 minute) must be performed as it contributes to the unfolding of haze-forming proteins and facilitates their enzymatic degradation by proteases, as well as leads to denaturation of the protease itself [115], Resolution OIV-OENO 541A-2021; Resolution OIV-OENO 541B-2021. In this context, a protease of *Botrytis cinerea* BcAp8 has been described to hydrolyse grape chitinases at moderate temperatures [116]. Also, evaluation of the effects of the joint use of heat treatment (75°C, 2 minutes) and application of proteases on the protein stability was recently studied by Comuzzo et al. [117]. These authors also evaluated the effect of the heat treatment with application of protease on the wine volatile composition and observed that the wines submitted to this treatment presented a lower content of esters produced during alcoholic fermentation and a higher concentration of esters that are characteristic of ageing such as ethyl lactate [117]. The potential of ultrafiltration (UF), in association with heat and proteolytic enzymes,

to eliminate haze-forming proteins and stabilise white wine was evaluated by Sui et al. [90]. Since the treatment with enzymes (proteases) to eliminate wine haze-forming proteins needs a previous thermal treatment to denaturant them, recently the application of ultra-high-pressure homogenisation (UHPH) was suggested as a possible alternative to the heat treatment. In this way, the application of UHPH could be in the future a new technological solution for using enzymes in the wine protein stabilisation process and probably with a lower impact on the wine volatile composition [118].

4.3 Fining and adsorption treatments

These practices include the use of adsorbents [117], such as zirconium dioxide (ZrO_2) also known as zirconia [37, 119–121] carrageenan [6, 92, 122], silica gel, hydroxyapatite and alumina [42], magnetic nanoparticles [123] zeolites [124, 125] and dicarboxymethyl cellulose [126]. However, all of them are at the moment under investigation and therefore not allowed by the International Organisation of Vine and Wine (OIV) or by the European Union (EU) legislation for application in wine.

Mannoproteins [127] are already allowed to be used by the OIV [115]. Chitin and chitosan [127, 128] have been authorised by the European Union (EU) for removal of contaminant and heavy metals, avoidance of turbidity and decrease of unwanted *Brettanomyces* spp. population (EU) 53/2011), but only chitin (Oeno 367-2009 Chitin-Glucan [115] and chitosan (Oeno 368-2009 Chitosan [115] from the cell walls of *Aspergillus niger* or *Agaricus bisporus* are allowed to be applied in wine.

In recent times, some researchers also studied the application of nanomaterials to remove unstable wine proteins [129]. Magnetic steel nanoparticles coated with acrylic acid have been experimented for the selective removal of pathogenesis-related proteins from wines by cation exchange mechanism due to the existence of carboxylic acid groups in the modified surface, and the results showed that they are highly efficient in decreasing haze-forming proteins [122, 130, 131]. Although these nanoparticles have been found to be effective in removing proteins in protein-unstable wines, their efficiency in wines seems to be affected by the low pH of wines that affects the cation exchange capacity of the nanoparticles due to the protonation of the carboxylic acid groups. Also, mesoporous nanomaterials proved to have high efficiency in decreasing haze-forming proteins with lesser wine aroma decrease compared with bentonite fining [132].

Wine-unstable proteins could also be adsorbed by zirconium dioxide [4, 119, 120, 133], a metal oxide usually known as zirconia, and consequently stabilise the wine by removing, especially, wine protein fractions between 20 and 30 kDa. Also, zirconium oxide pellets enclosed into metallic cage submerged in wine at 25 g/L for 72 hours stabilised white wines by removing unstable proteins with the advantage to be regenerated [37].

Results show that the water-insoluble dicarboxymethyl cellulose successfully reduced the wine protein content and turbidity, producing heat-stable wines with concentrations higher than 0.25 g/L [126].

Polysaccharides extracted from seaweeds were also studied by several researchers due to their negative charge at low pH, can electrostatically flocculate and precipitate positively charged proteins and remove wine unstable proteins [6, 122, 134]. Carrageenan uses at different winemaking stages were considered, and the application stage showed to be very important for its effectiveness [6, 92] More recently, Arenas et al. [17] showed that k-carrageenan reduced the content of pathogen-related proteins and consequently the wines protein instability, being even more efficient than sodium and calcium bentonites (**Figure 1**). On the other hand, these authors also showed that chitosan from fungal origin was unable to

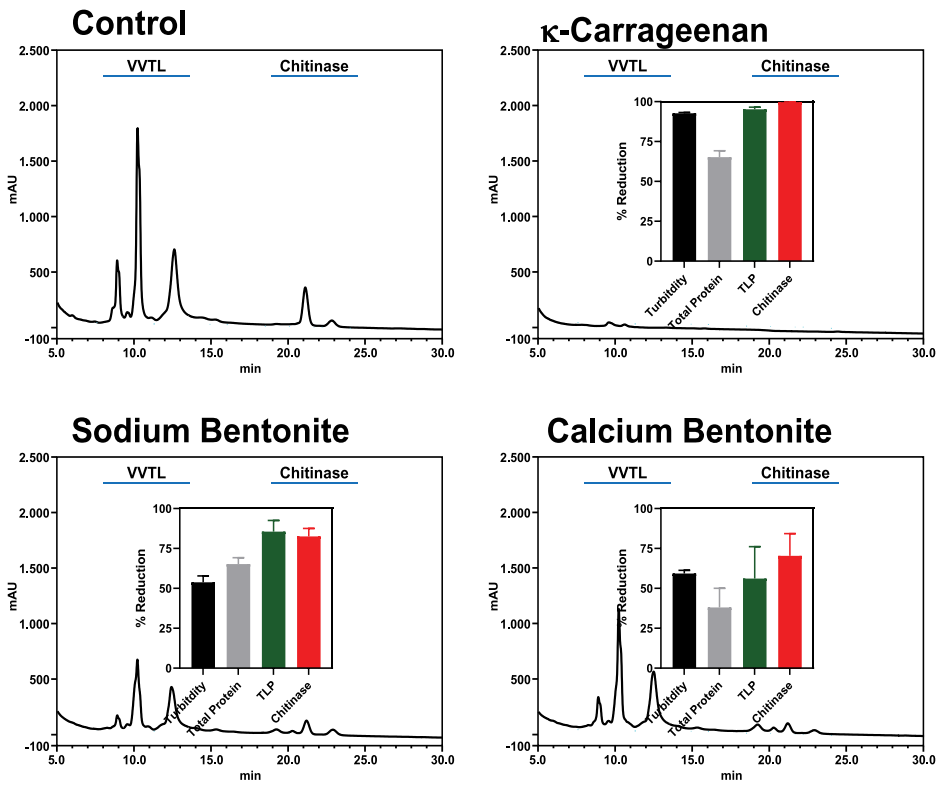

Figure 1.
Reversed-phase HPLC results and percentage reduction of turbidity (NTU), total protein (mg/L), Vitis vinifera thaumatins (VVL, mg/L) and chitinase (mg/L) for Albariño white wine produced without pre-fermentative skin maceration and the impact of the different products applied for its protein stabilisation. Control wine without any additive; after addition of k-carrageenan (100 g/hL); after addition of sodium bentonite (120 g/hL); after addition of calcium bentonite (120 g/hL). All chromatograms were acquired by analysis of a 5 mg/mL solution of the high molecular weight after elimination of the low-molecular-weight material by application of 6 M urea and repeated ultrafiltration through a 10 kDa cut-off membrane (adapted from Arenas et al. [17]).

heat stabilise the wines, and it was also observed that after the application of this oenological product, the levels of pathogen-related proteins remained unchanged. Additionally, the application of the fungal chitosan decreased the concentration of wine polysaccharides by 60%, as also observed after the application of sodium and calcium bentonite (16–59%). However, the application of k-carrageenan did not change the concentration of wine polysaccharides.

Chitin [135] and chitosan [128], polysaccharides mainly from *Aspergillus niger*, also have the capacity to decrease wine haze-forming proteins. It was observed that wine haze induced by the heat test is reduced by 50% after the addition of 1 g/L of chitin, while the addition of 20 g/L of chitin decreased the haze by 80%. The haze decrease perceived was related to the removal of the class IV grape chitinases [136]. Colangelo et al. [128] also showed that wines fined with 1 g/L of fungal chitosan-glucan enhanced heat stability at 55–62°C, and this was also due to the reduction of chitinases.

Mannoproteins existing in yeast cell walls have also been reported to have a protective effect on wine protein haze development [137, 138]. Waters et al. [54] showed that mannoproteins protect unstable wine proteins, avoiding wine turbidity when wine is exposed to high temperatures; these authors indicated that this action does not avoid the protein precipitation. Instead, they detected a reduction in particle size, justifying, in this way, the wine stabilisation observed when determined

by turbidimetry. However, their effectiveness for protein stabilisation is highly dependent on the mannoprotein structural characteristic, according to Ribeiro et al. [137], the effectiveness of commercial mannoproteins was related to their chemical composition, namely their high mannose-to-glucose ratio.

5. Final remarks

White wine protein instability has still been an important problem in the wine industry by the frequency of haze formation on the white and rose bottled wine. The grape variety and its grape sanitary conditions, 'terroir', the climate conditions during the grape maturation, the mechanical harvest and some winemaking operations could influence significantly the levels of unstable proteins in the wines. The principal proteins responsible for the protein haze are chitinases and thaumatin-like proteins, considered pathogenesis-related proteins (PR) with different thermostability and sizes. Many factors could affect their wine stability, such as wine exposition to high temperatures, wine pH variation, organic acids levels, metals composition, sulphur dioxide levels and the presence of phenolic composition and its degree of polymerisation. Some factors are yet unknown (X factors) but they influence protein precipitation. Even after many works that have been done in the last years, sodium bentonite has still been the most effective treatment to eliminate unstable proteins from white and rose wines. In fact, many products and treatments had been tested to remove these unstable proteins, such as proteases, different polysaccharides (chitin, chitosan, CMC, carrageenan), yeast mannoproteins, some of them show an interesting efficiency, such as carrageenan in a recent work. Finally, white wine proteins stabilisation has still been a problem for the wine industry, and it is necessary to continue developing new approaches to remove or mitigate this important problem. It is necessary to get new solutions to decrease the amount of bentonite used in the wine industry per year by those negative sensory impacts after wine treatment and by environmental concerns.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Luís Filipe-Ribeiro*, Fernanda Cosme and Fernando M. Nunes
Chemistry Research Centre-Vila Real (CQ-VR), Food and Wine Chemistry Laboratory, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

*Address all correspondence to: fmota@utad.pt

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Chapter 16

The Light Struck Taste of Wines

Ana María Mislata, Miquel Puxeu, Monserrat Mestres and Raúl Ferrer-Gallego

Abstract

The light-struck taste (LST) of wine is a defect that mainly occurs in bottled wines exposed to light. Factors that influence the onset of the LST in wines were reported. The effect of grapes and wine composition, the alcoholic fermentation process, the yeast strains used and the conditions of yeast nutrition were included. The external factors, such as bottle color, time and nature to light exposure and type of closure were considered. Finally, the analysis of the main molecules related to this default (sulfur volatile compounds and their amino acids and riboflavin precursors) and possible prevention measurements were also exposed.

Keywords: amino acids, wine, riboflavin, aroma, LEDs, stoppers, sensory analysis

1. Introduction

The light-struck taste (LST) of wine is a defect that mainly occurs in white and rosé bottled wines exposed to light for a considerable period of time. The light-induced changes in wines are mainly due to photochemical reactions but several factors can influence it. The most important are related to the wine composition, the spectrum of the light source, the intensity of the radiation, the optical properties of the glass bottle and the irradiation time. The wine composition alterations caused by these factors lead to detrimental effects on the sensory attributes. In bottled wine, the exposure to light can cause a significant browning effect and bring about unpleasant smells [1–5]. These bad effects were due to the photochemical oxidation involved in this deterioration which can affect phenolic substances, acids, alcohols and other wine compounds [6, 7].

In particular, riboflavin (RF) or vitamin B2 is one of the most important precursors in the generation of aromas related to the LST. This is a highly photosensitive molecule, which can undergo photochemical degradation through different ways. In addition, sulfur amino acids are involved in the photo-reduction of riboflavin being also important precursors in the appearance of sulfur volatiles. That is why, both methionine and cysteine (the sulfur amino acids of wine) in the presence of riboflavin (**Figure 1**) can suffer photo-oxidative degradation giving raise to unpleasant aromatic volatile sulfur compounds (VSCs), such as hydrogen sulfide, methanethiol, dimethyl sulfide and dimethyl disulfide (**Figure 1**). The combination of these aromatic sulfur compounds leads to the defect called 'light struck taste', 'taste of light' or in French 'goût de lumière'. Wines with this default presents unpleasant aromas described as rotten egg, garlic, onion, boiled cabbage and sometimes also provides a metallic taste perception. Given the importance of this defect and the economic losses that it may entail, both oenological and photovoltaic strategies are

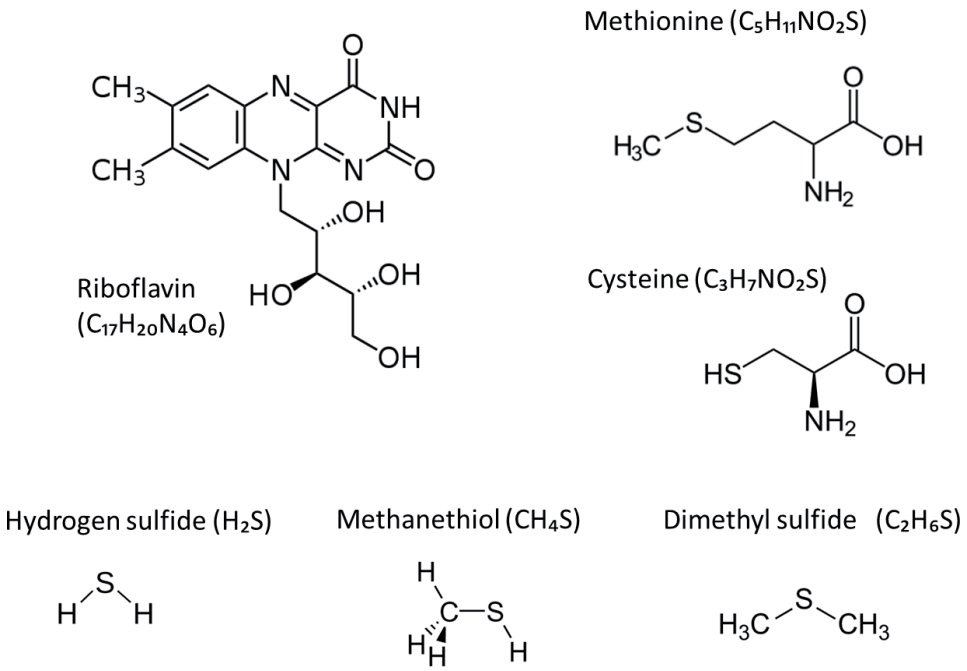

Figure 1.
Molecular structures of precursors and aromatic compounds related to the LST default.

currently being sought to prevent the appearance of this default in bottled wines stored in wineries, supermarkets or wine bars, in order to offer consumers an optimal wine quality.

2. Influencing factors of the light-struck taste

2.1 Grape and juice composition

The grape is made up of a large amount of nutrients which will pass into the must once it is crushed which will be able to participate in the formation of the LST of wine. The major components of the grape are sugars, mainly glucose and fructose, which will be transformed into alcohol during fermentation. Nitrogen is an abundant component in grape juice with content around 200–300 mg/L. It appears in two differentiated chemical forms: inorganic (basically as ammonium form) and organic (made up of amino acids, peptides and proteins). The nature and concentrations of amino acids in grapes depend on a wide range of factors, such as fertilization, climatic conditions, and grape variety [8, 9]. They are consumed by yeasts during alcoholic fermentation and can produce some positive volatile compounds, such as esters, or negative ones, such as sulfur volatile compounds, which will influence the final aroma of the wine [10]. Amino acids represent up to 40% of the total nitrogen in wines, and yeasts release some amino acids at the end of fermentation. They act as aromatic precursors through different chemical reactions and to form aromatic compounds. On the other hand, among the minority compounds in wine related to LST, riboflavin should be highlighted. This components is found at very low concentrations (between 3 to 60 μg/L) in grape must [11]. The formation of this compound is related to the *Saccharomyces* metabolism that will play a very important role in the formation of reduction aromas of wine. Finally, sulfur aromatic compounds occur more frequently in wines from vineyards

planted in alkaline soils. This is because high pH of soil makes difficult to absorb copper which, normally used as fungicide treatment, helps to eliminate the sulfur compounds produced during winemaking. However, the current trend to replace this metal in new fungicide formulations could lead to an increase in the content of sulfur compounds in wines and therefore the risk of LST appearance. Moreover, the use of sulfur-rich phytosanitary products used in the vineyard may lead to obtaining musts with certain risk of producing this defect.

2.2 Wine fermentation, yeast and nutrients

2.2.1 Nitrogen

Yeasts use the nutrients of the must for their growth during fermentation process. Here, nitrogen is essential to develop reactions that will derive in the formation of secondary metabolites which are very important on the quality of the wine, such as glycerol, organic acids (lactic, acetic, succinic), esters, sulfur compounds or amino acids released during this phenomenon. Usually, during the alcoholic fermentation, nitrogen is added during the exponential phase of fermentation that corresponds to the growth of the yeast (first days of fermentation). Nitrogen can be assimilated by yeasts during winemaking in two different forms, as ammonium or as amino acids. In this phase it has an effect on cell growth and on the rate of fermentation [12]. In musts with few nutrients, the amount of assimilable nitrogen drops early and induces the production of hydrogen sulfide (H_2S) due to the absence of compounds that capture sulfur.

Wine yeasts can form H_2S from inorganic sulfur compounds (sulfate or sulfite), or organic sulfur compounds (cysteine, methionine or glutathione). The production of H_2S can occur from the Sulfate Reduction Sequence (SRS) route, where sulfate is used for the biosynthesis of cysteine and methionine. Sulfate is accumulated from the medium, and then reduced to sulfite following sulfite is reduced by sulfite reductase to sulfide. Therefore, when dealing with nitrogen-deficient musts, yeast will tend to synthetize it from nitrogenous precursors *o*-acetilserina and *o*-acetilhomoserina, with which the sulfite produced will be excreted as hydrogen sulfide (H_2S) [13]. Therefore, in wines with a limited content of nitrogen the supplementation with sulfur amino acids the production of hydrogen sulfide can increase considerably. H_2S is a highly reactive compound, and it can combine with different components present in wine forming other VSCs [14]. Mercaptans, sulfides and disulfides can be also found in wines.

In many cases, H_2S production can be controlled by adding nitrogenous salts such as diammonium phosphate (DAP). Some studies suggest that a concentration of 200–250 mg/L of assimilable nitrogen is necessary to minimize the risk of H_2S production. However, not all commercial strains show the same behavior to the improvement of the must by the addition of diammonium phosphate, and usually indicates a deficiency in the juice of one or more vitamins, pantothenic acid, pyridoxine or biotin, which is involved in the metabolism of H_2S. The persistence of H_2S production problems, even with nutrient supplementation, requires the selection of yeast with low H_2S production in such musts.

It has been reported that some strains appear to produce H_2S inherently without being affected by environmental conditions, possibly indicating a metabolic defect [15, 16]. Therefore, the H_2S production capacity of a specific strain has a genetic influence, since the H_2S production of different strains varies under the same conditions [13, 16, 17]. The excessive production of hydrogen sulfide that takes place during the fermentation process is a fairly common problem in winemaking [13, 17]. As mentioned, the persistence of H_2S production problems,

even with nutrient supplementation, requires the selection of yeast strains with low H_2S production. New yeast strains have been developed to produce undetectable amounts of H_2S [18]. In summary, yeasts and nutrients, such as the nitrogen content have a manifest influence on the different metabolites produced during fermentation, many of them with a very clear impact on the wine aroma and therefore in the LST default.

2.2.2 Riboflavin

Riboflavin acts as a photosensitizer in many foods and beverages. The RF level in grapes is usually less than a few tens of micrograms per liter of must [19], but can increase during winemaking mainly due to the metabolic activity of *Saccharomyces cerevisiae* [20]. Values close to 150 µg/L or even higher can eventually occur in wine depending on the yeast strain used for the alcoholic fermentation [21, 22]. Riboflavin-producing yeast strains have occasionally been found to be methionine-producing as well, which may increase the risk of spoilage [21]. The amount of methionine oxidized in wine exposed to light is related to several physical and chemical factors, including the concentration of riboflavin, oxygen, and other amino acids. Photosensitized RF can oxidize methionine as well as other amino acids. The reduced riboflavin can then be oxidized back to riboflavin by oxygen [23]. It is also known that the presence of riboflavin in wine is mainly due to the metabolism of the yeast *Saccharomyces cerevisiae*. Some *Saccharomyces* strains can prevent a high amount of riboflavin in wine [21]. Yeast is known to contain a gene, RIB5, which encodes the formation of the enzyme riboflavin synthase, which is involved in the last step of RF synthesis by yeast [20]. The use of yeast strains that have a lower capacity to produce riboflavin may be a potential means of minimizing its concentration in wine.

Some studies carried out at our facilities in the Wine Technology Center (VITEC) reported the importance in the use of different yeasts and nutrients to carry out the fermentation to diminish the RF content in wine (**Figure 2**). Different types of commercial *Saccharomyces cerevisiae* strains were assessed with different types of nutrition during fermentation. In this case, one of the *S. cerevisiae* strain used (yeast strain 2) produced higher RF content in three of the four studied nutrition conditions. In addition, nutrition 1 and especially nutrition 3 increased noticeably the production of RF. This could be explained by differences on the metabolism of each strain and the characteristics of the nutrients. These two conditions of nutrition were based on yeast cell walls, richer in vitamins while nutrition 4 was based in inorganic addition by DAP.

The ability of certain oenological yeast nutrients added during fermentation generally used to prevent the stop or sluggish fermentation can release RF. Yeast

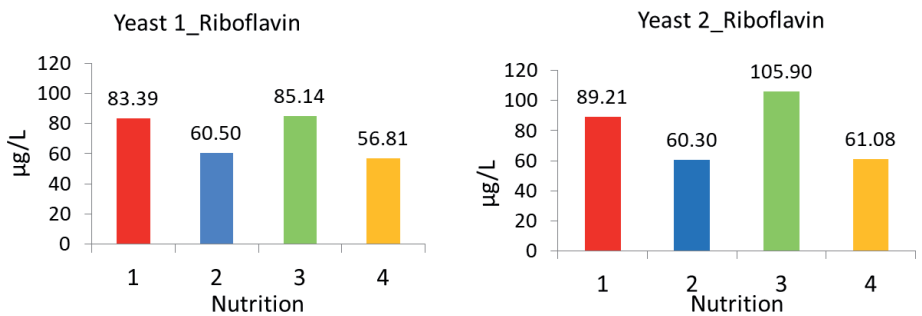

Figure 2.
Production of riboflavin with two different strains of Saccharomyces cerevisiae *through four different types of nutrition.*

extract-based nutrients often contain vitamins, including RF, which can therefore increase its amount content in wine. The use of low RF products has been proposed to prevent the formation of volatile sulfur compounds. Yeast lysate can also be used as an additive to prevent the anti-fermentative activity of medium chain fatty acids [19]. The lipid fraction naturally found in yeast lysate may have affected the ability of fermenting yeast to produce purines, the precursors of riboflavin in yeast metabolism [24, 25].

2.3 Wine composition

2.3.1 The aromatic precursors

Riboflavin present in wine is likely the most important precursor of the light struck taste default. In food and beverages, riboflavin is naturally present as flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and riboflavin (RF). In wine only RF form has been detected [26]. In bottled wines, RF participates in light-induced reactions that affect changes in volatile compounds, color, and flavor [4, 26]. When the RF concentration in wine is greater than 100 μg/L, the wine is considered to have a high risk of presenting the LST [27]. RF is a highly photosensitive compound which can be degraded in the presence of fluorescent or phosphorescent light, with wavelengths ranging from 370 to 450 nm. Its photochemical degradation can follow several paths of which intermolecular photo-reduction is the most relevant. The first step in the degradation mechanism is the uptake of a pair of electrons from an external donor (in this case methionine) by riboflavin. By this way a reduced flavin and methional is obtained. Methional is extremely unstable and breaks down to form methanethiol and acrolein. And the reaction of two methanthiol molecules can produce dimethyl disulfide [28]. The interconversion of diethyl disulfide and ethanethiol in presence of sulfites was also reported [29]. Although the rate of reaction is slow at wine pH, model predictions indicate that the reduction of diethyl disulfide to ethanethiol over time can be of sensory importance in wine [29].

RF plays a fundamental role in the oxidation of sulfur amino acids such as methionine and cysteine. Strecker's degradation of amino acids such as methionine and cysteine to aldehydes by α-dicarbonyl compounds formed during fermentation or oxidation contributes to the evolution of the aroma in bottled wine [30]. Glyoxal, and α-dicarbonyl compound generated during alcoholic and malolactic fermentation, reacts with methionine to form methanethiol and dimethyl disulfide, and with cysteine to form hydrogen sulfide, methanethiol, and other compounds [31, 32].

Wine is made up of a large number of different amino acids and, among them, methionine and cysteine are also important precursors for the LST appearance as these have sulfur atoms in their structure (**Figure 1**). Maujean (2001) described the thermal origin of volatile sulfur products in Champagne wines stored at 25°C in the dark could be formed by Strecker degradation of these sulfur amino acids [28]. Strecker's degradation of amino acids such as methionine and cysteine to aldehydes by α-dicarbonyl compounds formed during fermentation or oxidation contributes to the evolution of aroma in bottled wine [33]. Glyoxal (α-dicarbonyl compound generated during alcoholic and malolactic fermentation) reacts with methionine to form methanethiol and dimethyl disulfide, and also reacts with cysteine to form hydrogen sulfide, methanethiol, and other compounds [31, 32]. Methional, the initial product of Strecker's degradation of methionine, could be degraded via (retro-Michael mechanism) to form methanethiol, which is then oxidized to dimethyl disulfide [32]. Singlet oxygen, produced in photosensitized reactions, reacts with methionine resulting in the formation of dimethyl disulfide [34].

Maujean (1984) proposed that in white wine exposed to light, triplet riboflavin oxidizes methionine to methional, which then degrades to form methanethiol and dimethyl disulfide [35].

In relation to the amino acids degradation and the consequently formation of sulfur volatiles, the studies carried out in our laboratory (VITEC) confirm the data found in literature. **Figure 3** shows white wines bottled in clear glasses and exposed to three types of LED lights with different wavelength emissions. The concentrations of methionine, cysteine and the volatile sulfur compounds (as the sum of hydrogen sulfide, methantethiol, dimethyl sulfide and dimethyl disulfide), were determined after keep the wine in darkness, and after being exposed for 6 and 240 hours to different sources of light (L.1, L.2 and L.3). Results showed that the longer the light exposure time the lower the concentrations of the amino acids studied, and the higher the formation of volatile sulfur compounds. Furthermore, it should be noted that L.1 was the light that caused the greatest degradation of cysteine and methionine. As mentioned above, the nature of light is an important factor that can favor the LST default. In the next section, we can observe some examples.

2.3.2 The volatile composition

The LST is related to the formation of volatile sulfur compounds. Hydrogen sulfide, methanthiol, dimethyl sulfide, and dimethyl disulfide appear to be largely the main compounds responsible for the occurrence of this default [28]. All these compounds are mainly responsible for the formation of "reducing" aromas after bottling [30, 33, 36]. These compounds are characterized by unpleasant aromas in wines. On the one hand, within the thiol family is found hydrogen sulfide, which is a characteristic compound for providing wines with unpleasant aromas of rotten eggs, decomposing algae or wastewater. Other characteristic thiol of this defect is the mentioned methanthiol that contributes by descriptor aromas related to putrefaction smell and cooked cabbage. It should be noted that both compounds present a very low odor threshold (OT) values, corresponding to 1.6 µg/L and 0.3 µg/L respectively [37, 38]. On the other hand, within the family of sulfides and disulfides are found dimethyl sulfide and dimethyl disulfide, characteristic compounds for providing aromas associated with cabbage, asparagus, corn or onion flavors when present in high concentrations. They have an odor threshold around 25 µg/L and 29 µg/L, respectively [39–41]. The emergence of sulfur compounds related to the LST usually is also linked to a loss of fruity aromas of wines, such as ethyl and acetate esters, alcohols and fatty acids [42].

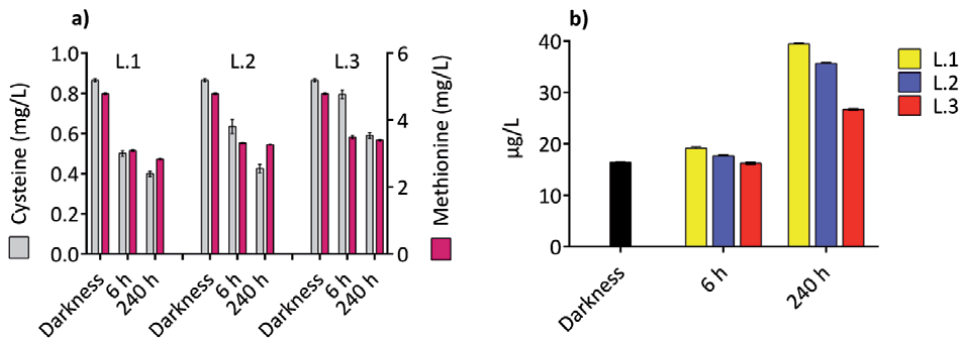

Figure 3.
a) Sulfur amino acid content (cysteine and methionine) and b) volatile sulfur content, in white wines bottled in clear glass after being exposed to three types of LED lights (L.1, L.2, and L.3) during time (6 and 240 hours) compared to controls in the darkness.

Moreover, the volatile sulfur compounds related to the LST of white wines is influence by the color of bottle. Here, some example comparing green bottles and clear bottles are shown (**Figure 4**). The types of source of light were also evaluated comparing six types of LEDs (**Figure 4**).

As can be seen in **Figure 4**, the white wine bottled in clear glasses presented higher concentrations of reduction aromas after 10 days of exposure with the LA, LC and LE lights, while the wine with the green bottle presented the highest concentrations with light LA. This is consistent with the degradation of riboflavin. The greater the degradation of riboflavin, the greater the presence of aromas of sulfur compounds in the wines (see Section 2.6). All this is due to the innovation of new LEDs which minimize or eliminate the emission of the region between 370 and 442 nm of the spectrum, thus reducing the risk of wine degradation (see Section 2.5 and 2.6).

2.4 Type of closures (OTR)

The last step in winemaking process is bottling. The main aim of this is to package the wine to get the customers in comfortable and attractive way and also to preserve the organoleptic characteristics of wines. Although it may seem the easier process step, it is critical to maintain and, sometimes, also to improve the qualities of the product over time until the consumption. On the one hand, wine should be prepared and fined to prevent chemical precipitations of salts, color matter, protein haze and microbiological alterations as well, always respecting the nature of the wine and their characteristics. On the other hand, the type of closure should be selected according the consumption time expected. Closures should assure that the contents do not drip out of the bottle and that the contents were not altered by oxygen. Nowadays, wine producers have several options to stopper wine bottles, such as screw caps, crown corks, plastic caps of grass closures with plastic sealing.

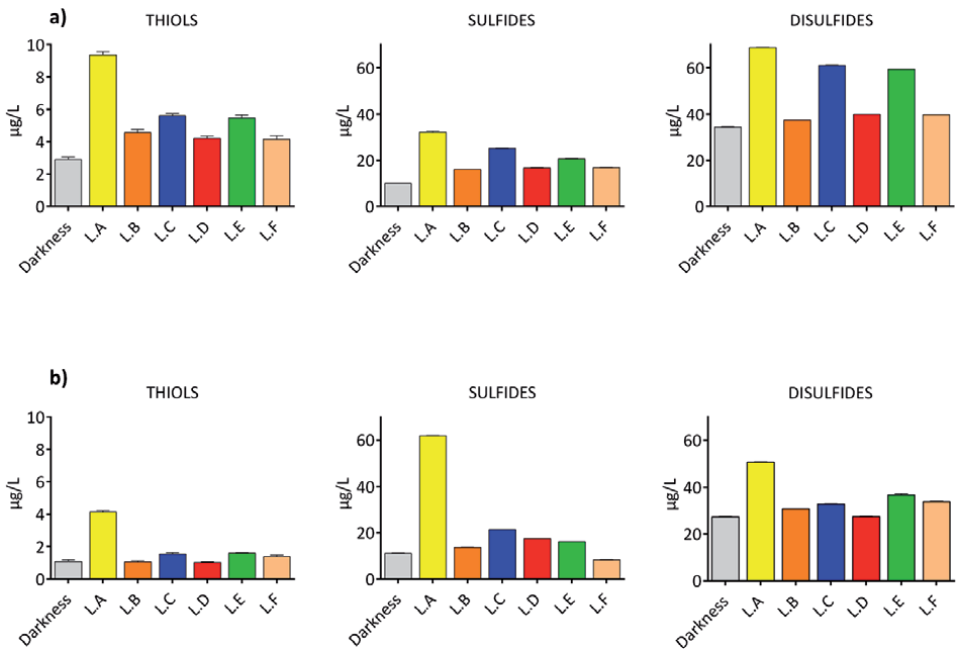

Figure 4.
Production of volatile sulfur compounds in white wines bottled in different color of bottles (clear bottles; a) and green bottles; b)) after 10 days of exposure with six different types of LEDs compared with darkness condition.

Several changes can take place in wine after bottling, some of them desired and expected as increasing of complexity, roundness, pleasant and desired evolution. Anyway, also unexpected changes derived from stoppers can also occur due wine oxidation or reduction [43]. Some of these unexpected changes can modify the quality of wines and, in the worst case, these wines could be considered defective products and often undrinkable.

One of the factors with more influences in wine aging and evolution is oxygen. Oxygen is trapped in the headspace of the bottle after bottling, it is present in the wine dissolved and also permeates through the closure combined with temperature and light, modifying the oxidative status of the wine during storage [44]. So, winemakers have the option to modify and control the evolution of their wine after bottling selecting the closure type. The flow of oxygen able to pass through the closure of a wine bottle is referred to as OTR (oxygen transmission rate in 24 h). This parameter depends on the thickness of the material and the partial pressure gradient between the atmosphere of the external environment and the headspace of the bottle [45]. This oxygen ingress is typically slower than the rate of oxygen consumption of the wine, so that, after consumption of the initial excess of oxygen, dissolved and headspace concentrations of oxygen are usually very low (often micrograms per liter) [46]. Other common indicator in oenology is the total package oxygen (TPO) which can vary over a range of approximately 1 and 9 mg/L. This parameter consist of the sum of two components, wine dissolved oxygen and headspace concentration [33].

Different OTR ranges could be found in bibliography, detailing the approximately oxygen transmission rate for each type of closure. Screw cap saranex and screw cap saran tin are the closure options with lower OTR values with 0.0006 and 0.0008 mL of O_2 per day, respectively (AWRI measurements) [47]. Micro agglomerate technical corks with a very low OTR could get similar values as screw cap close to 0.0006 and 0.0007 mL of O_2 per day, natural corks increase slightly the permeability till between 0.0002 and 0.006 mL O_2 per day (Jim Peck's MOCON measurements) Finally extruded synthetic closures showed a higher permeability around 0.0019–0.0030 mL O_2 per day (Jim Peck's MOCON measurements).

In the studies carried out at VITEC, the volatile composition responsible for the reduction aromas was evaluated taking into account the use of five corks (from C1 to C5) with different OTR values (OTR values from C1 to C5 was of lower a major) in sparkling white wines throughout 3, 6 and 12 months of aging in bottle. A crown-cap (CAP) was used as control wine (**Figure 5**). As can be seen in this figure, the control samples with CAP stoppers and C1 corks with the lowest oxygen transmission (OTR), were the ones with the highest concentrations of thiols, sulfides and disulfides, mainly after 12 months (12 M) of aging in the bottle.

2.5 Type of bottles

As stated previously (**Figure 4**), another very important factor in the wine bottling step is the choice of the container in which the wine will be stored until its consumption. This container or packaging must take into account the protection and conservation of the product. In the case of wine there are different types of containers such as the tetra brick which is a cardboard for drinks made up of different layers of polyethylene, paper, and aluminum; the bag in box consisting of a polyethylene bag and a tap with a valve for dosing it; PET (polyethylene terephthalate) plastic containers; aluminum cans type; and finally, the most widely used container in the world in the case of wine, glass. Which is a mineral product obtained by fusion and that solidifies without crystallizing. It is also an inert material, and from an environmental point of view it is favorable because it is a fully recyclable material.

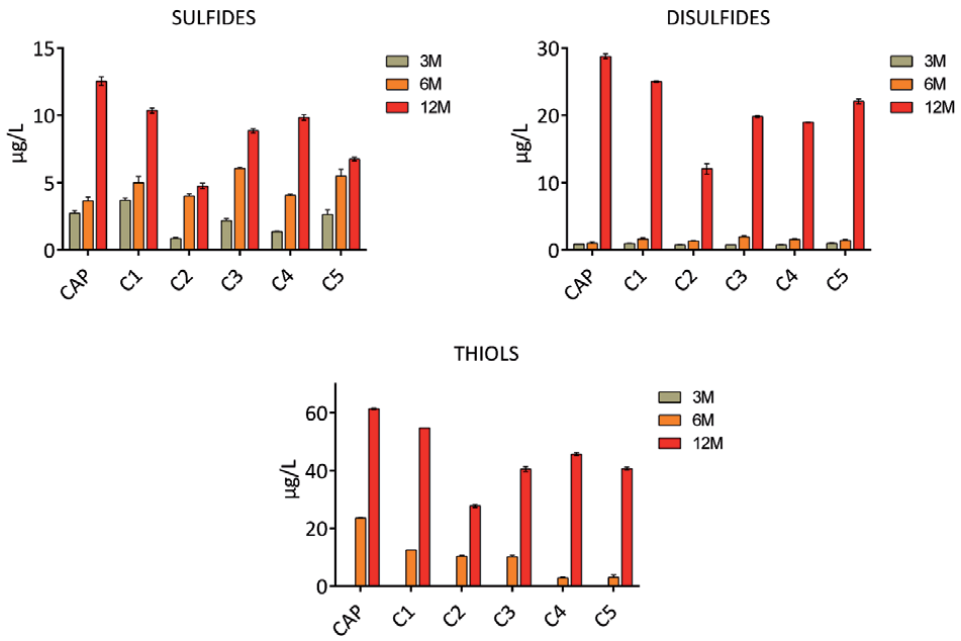

Figure 5.
Concentrations of thiols, sulfides and disulfides in sparkling white wines stopped with 5 different types of corks and cronw-CAP (CAP), over 3, 6 and 12 months of aging in the bottle.

Nowadays, different shapes of glass bottles are used (Bordeaux, Burgundy, Champagne, Rhin, Jerez, Porto) with different capacities, and of different color and shade of glass (flint, amber, green, blue, etc). The choice of the color of the glass is of great importance with regard to the preservation of wine during storage or aging. This is due to the fact that the incident light in the bottles penetrates through the glass, producing oxidation–reduction reactions in the wine and consequently affecting its organoleptic qualities. Glass wine bottles only transmit wavelengths greater than 300 nm [6]. Standard clear bottles (flint) generally transmit more than 80% of visible UV radiation above 360 nm, while clear bottles with additional UV protection, which are made by adding a UV absorbing species to glass or by coating clear bottles with a film that contains a species of this type, transmits less UV radiation [48]. Green bottles transmit considerably less light than clear ones, particularly in the region below 520 nm, while amber bottles transmit very little radiation below 520 nm. For the darker colored bottles, the heavy bottles, which have thicker glass, transmit slightly less light than the lighter counterparts.

2.6 Aging and time of light exposure

It is well known that wine is very sensible to temperature which can directly affect their global quality [49]. Temperature can play a significant role impacting directly to the color, aroma and mouthfeel accelerating their natural aging process. Ideally, wines should be stored in conditioned rooms in cellars normally with air conditioned facilities (15–20°C). However, wine could experiment changes in their temperature being exposed to less optimal conditions, especially during transport or storage distribution process [50]. Visual affectations could also be observed, being the most notable the formation of a haze resulting from the denaturing of proteins in rosé and white wines [51]. If temperatures are so high and wine it is packaged in glass bottles the temperature effects can include cork push due to the volumetric expansion of the wine impacting directly to closure seal integrity and

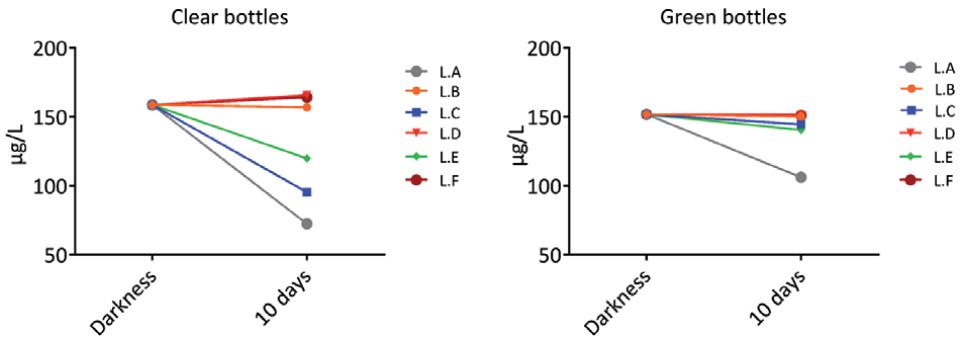

Figure 6.
Degradation of riboflavin in a white wine bottled in a clear bottle and in a green bottle after 10 days of exposure to 6 different LEDs.

impacting in oxygen transmission rate (OTR) [52]. Several published studies detail the changes showed by wines when these are exposed to high temperatures, and other composition variables as pH, SO_2 concentration, alcohol content, tannins concentration or others [3, 37, 53–58].

Several authors as studied the direct effect of temperature on the volatile composition of red and white wines [59]. Over a 21-day period, the study found that a constant 40°C heat treatment had a greater impact on the aroma and volatile composition of the wines compared with that of the 20°C/40°C cycled treatment, which in turn had a greater impact on that of wines stored at a constant 20°C. These studies conclude that it is evident that as a direct result of heat, the fruity acetate compounds in the wines are disappearing and aged-like characters have developed. Temperature not only affects the volatile compounds, also non-volatile compounds as polyphenols experiment changes at high temperature. The most common effect on non-volatile in red wines is a decrease in anthocyanin concentration and a corresponding increase in tannin-bound anthocyanins.

Apart from the temperature, the light incidence is also an important factor to take into account during the aging period. As mentioned above, the susceptibility of white wines to produce the LST has been mainly associated with the photosensitizer riboflavin and the produced unpleasant odor has been attributed primarily to volatile sulfur compounds. Maujean found that hydrogen sulfide, methanethiol, and dimethyl disulfide were formed in Champagnes exposed to a solar simulation lamp in glass cuvettes, in the absence of oxygen [28]. In our study (**Figure 6**), the degradation of RF was found just after 10 days of exposure to light in white wines bottled with green and clear bottle body. As can be seen in **Figure 6**, the white wine with a clear bottle presented large decreases of RF. More than 50% of the initial content in clear bottles and more than 30% in gree bottles in the case of L.A. The white wine bottled in clear glass showed a degradation of riboflavin with more types of lights.

3. Analysis of the main chemicals related to LST

To evaluate the aromatic defect that we are dealing with, it is necessary to know which compounds could be responsible but, to know the contribution of each one to wine aroma, it is also necessary to know their concentration. In addition, to have a better control of this problem, it would also be very interesting to obtain information on the concentration of the precursors since their degradation provides the unwanted volatile sulfur compounds (VSCs). However, since from a physicochemical point of view the precursors are chemical compounds very different from

the VSCs, both the sample preparation techniques and the analytical techniques will be also different in each case.

3.1 Analysis of aromatic precursors

3.1.1 Riboflavin

Riboflavin, also known as vitamin B2, is a dimethylated isoalloxazine linked to ribitol. In fermented beverages like wine, this heterocyclic ring is mainly found as free riboflavin although few amounts of the mononucleotide and dinucleotide forms can be also found. This is why some analytical methods propose to convert these forms to free form prior the analysis and to quantitate the total riboflavin content [26]. Traditionally, this compound has been determined by microbiological or fluorimetric methods [60]. However, when dealing with complex samples such as wine, the high performance liquid chromatography with reversed-phase column (RP-HPLC) is the most suitable technique as it separates the riboflavin from interferences. Among the different possible detectors including UV/vis, mass spectrometry and fluorescence, the latest is the most used because riboflavin naturally fluoresce. This property allows the injection of the sample directly into the HPLC although a sample filtration is recommended to avoid light scattering effect [26, 61]. Finally, it should be noted that some alternatives to these expensive and time-consuming HPLC methods have been developed. One of them is based on the fluorescence quenching effect produced by the riboflavin-binding protein what is measured by using a single diode fluorimeter [62]. According to the authors, the results obtained are comparable to those obtained by HPLC methods. The other alternative involves the use of UPLC which has become the modern HPLC showing higher sensitivity and chromatographic efficiency with a consequent run-time decrease [63].

3.1.2 Amino acids

Although the light-struck aroma precursors are only cysteine and methionine, the analysis methods found in the literature do not focus solely on these amino acids but consider as much of them as possible. Among the different techniques found in literature, the HPLC is the most frequently used for the determination of these compounds in wine or must. However, since the amino acids have no a specific chromophore group to be detected, a derivatization step is necessary. Although this derivatization can be performed before or after chromatographic separation, the pre-column option followed by HPLC or UPLC has been more widely used due to its simplicity and versatility. This derivatization reaction can be performed by using several reagents which lead to derivatives detectable by different detectors. Thus, when using ultraviolet detector, derivatising reagents such as phenylisothiocyanate, diethyl ethoxymethylenemalonate or dansyl chloride can be used. The latter reagent can also be used with fluorescence detectors in addition to other such as *o*-phthaldialdehyde, 9-fluorenylmethylchloroformate or 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. All of them present some advantages and some drawbacks but, in all cases, the main problem is that the time spent on the analysis is long [8, 64]. This is why some authors have focused on developing faster methods, such as the use of a fully automated in-loop derivatization procedure [65]. However, although these methods have been successfully applied, the way to drastically reduce the analysis time has been achieved when the derivatization step has been avoided. Today the only technique that allows a high degree of sensitivity and selectivity in the determination of amino acids without derivatization is the

so-called liquid chromatography with tandem mass spectrometry (LC–MS–MS), a mass spectrometer system highly specific for each compound structure. However, it should be pointed out that this costly technique has been applied very little to the analysis of wine components, and even less to the amino acid analysis of wine.

3.2 Analysis of volatile compounds

The volatile sulfur compounds related to LST constitutes a chemical family that includes thiols, sulfides and disulfides. This structural diversity together with the highly reactive nature of these compounds, their low volatility and their low concentration in a matrix as complex as wine, make their analysis considerably difficult.

Although the older bibliography references show methods developed as early as the 1990s which used sulfur-specific ion electrodes or the spectrophotometry with previous treatments to trap sulfur compounds but these methods have been rendered obsolete [66, 67]. In fact, nowadays, the best results are obtained when using gas chromatography coupled to specific detectors so this is the most widely used technique to analyze these compounds. Flame-photometric (FPD) [68], sulfur chemiluminescence (SCD) [69] and more recently pulsed-flame-photometric [70] are the usual required detectors. The use of the mass spectrometry (GC–MS), even being a nonspecific detection system, can be a good option mainly when working with SIM mode as it confers better sensitivity.

In any case, taking into account the usual low concentrations and the highly reactivity of VSCs, a preconcentration technique with minimal manipulation of the sample is required prior to chromatographic separation. Thus, while liquid–liquid extraction systems (either with vacuum or using reagents that selectively trap thiols such as pHMB) have not been very successful, the application of the headspace technique has given very good results. It should be noted that the concentration process required is only achieved with the dynamic modality of this technique which is also called purge and trap technique. Among the different traps, the best results are obtained when working with cold traps because, when dealing with chemical traps and complex matrices such as wine, the so-called memory effect usually occurs due to the difficulty of cleaning the traps between analyses. More recently, the technique that has emerged as the most appropriate is the so-called solid-phase microextraction (SPME) which is applied to the headspace of the sample wine. This simple and fast technique involves immersing a polymer-coated fiber into the headspace sample to extract and concentrate the analytes on the fiber. The fiber coatings that provides the best results on the sulfur compounds extraction have been Carboxen/polydimethylsiloxane or divinylbenzene/Carboxen/polydimethylsiloxane. Regarding the variables that influence the extraction process, the literature indicates that it is necessary to increase ionic strength with sodium chloride or magnesium sulfate, to agitate the sample with slow-medium speed and to use extraction temperature and time between 35 and 40°C and 20 and 40 minutes, respectively [37, 68].

4. Prevention and correction measurements

Up to now, different preventive and corrective measures have been studied to avoid the onset of the LST in both still and sparkling wines. Thus some proposed preventive measures are: avoiding grapes treated with sulfur on dates close to the harvest, avoiding excessive sulfite in grape juice, use yeast strains and nutritional conditions with low production of aromatic precursors of VSCs, racking wines

correctly, use micro-oxygenation or use preventing agents, such as polyphenols [5, 21]. Regarding to the possible corrective measures to reduce or eliminate VSCs, these could be: aerating the wine, using colloidal copper or copper sulfate or using insoluble absorbing materials such as bentonite, active carbon, or charcoal. In any case, whatever preventive or corrective measure has been chosen, it must be carefully applied as he inadequate practice of these treatments can lead to a great loss of the organoleptic characteristics of wines.

5. Conclusions

Several factors influence the formation of compounds responsible for the light struck taste (LST) in wines. As explained, the chemical composition of grapes and wines, the yeast strains used during the alcoholic fermentation process and their nutrition are decissive in the synthesis of their precursors and, subsequently to the concentration level of the VSCs. Moreover, other external factors are also crucial during the wine aging and storage period. The bottle color, the type of closure, the nature of light and the time of exposition seems to be the most important ones. As the light struck sensory perception depends on the amounts of VSCs, their quantitation becomes essential. Due to their low concentrations and the great complexity of the wine matrix, several instrumental analytical methods have been developed in recent decades to improve the analysis efficiency of these compounds. Finally, it should be noted that although the LST defect can be corrected several practices, its prevention is the best way to ensure the organoleptic quality of wine.

6. Future perspectives

The new trends in wine industry used to minimize the LST formation are related to the improvement of new LEDs technologies. These make it possible to use specific radiation sources that avoid the wavelengths most linked to the photodegradation of wine that causes this defect. Moreover, new stoppers with absorbing capacity of VSCs generated into the bottle and new materials for the packaging of wines are being developed [42, 71].

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Conflict of interest

The authors declare no conflict of interest.

Author details

Ana María Mislata[1,2], Miquel Puxeu[1], Monserrat Mestres[2] and Raúl Ferrer-Gallego[1*]

1 VITEC – Wine Technology Center, Tarragona, Spain

2 Instrumental Sensometry (i-Sens), Department of Analytical Chemistry and Organic Chemistry, Campus Sescelades, Universitat Rovira i Virgili, Tarragona, Spain

*Address all correspondence to: raul.ferrer@vitec.wine

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Chapter 17

State-of-the-Art Knowledge about 2,4,6-Trichloroanisole (TCA) and Strategies to Avoid *Cork Taint* in Wine

Andrii Tarasov, Miguel Cabral, Christophe Loisel, Paulo Lopes, Christoph Schuessler and Rainer Jung

Abstract

Cork stoppers have been used for many centuries to seal wine in various vessels. Therefore, corks have become a traditional part of wine packaging in many countries and still play an important role for the entire wine industry. Nowadays, there is a wide option of bottle cork stoppers on the market, such as natural corks, agglomerated and technical stoppers (1 + 1), etc. These cork closures have a number of advantages, including positive sustainable and ecological aspects. Natural cork material can also be responsible for *cork taint*, which imparts musty/moldy or wet cardboard off-odors to the wine. However, corks are not the only source of *cork taint* in wine, as will be shown in the present chapter. Over the past decades, a number of compounds have been detected that can contribute to the *cork taint*. Among them, haloanisoles play a major role, in particular 2,4,6-trichloroanisole (TCA), which has been shown to be responsible for 50–80% or more of musty defect cases in wine. Currently, the cork and wine industries have developed a number of tools and technologies to effectively prevent *cork tait* in wine or to remove it if the wine is already contaminated. These practical as well as analytical questions about the TCA defects are the subject of the actual chapter.

Keywords: 2,4,6-trichloroanisole (TCA), cork taint, musty, moldy, cork stopper, wine

1. Introduction

1.1 General information about *cork taint* and TCA in wine

The problem of *cork tainted* wines has been known to winemakers for a long time, but in the second half of the twentieth century, it began to attract more and more attention [1–3]. The origin of this problem was not well understood until the 1970–80s, before works on 2,4,6-trichloroanisole (TCA) and its contribution to the *cork taint* were published [4–6]. Now it is well known that TCA can migrate from cork stoppers and contaminate wine during bottle storage. Moreover, it was discovered that TCA is a widespread pollutant, which has also been found in various food products (coffee, poultry, etc.) as well as in water for public consumption.

Medium	Threshold level, ng/l	References
Wine	1.4[b]	[8]
Still white wine	1.5[c]	[9]
White wine	2.1 (3.1)[b]	[10]
Dry white wine	4[a]	[11, 12]
White wine	4–10[a]	[13]
Wine	10[a]	[14]
Red wine	22[a]	[15]

Mode of evaluation: [a]orthonasal; [b]retronosal; [c]unknown.

Table 1.
Sensory threshold levels for TCA in wine (adopted from [7] and modified).

TCA causes sensory defects, which are usually described as musty, moldy, and wet cardboard off-odors. The situation with TCA contamination is particularly challenging because even trace amounts of this compound can lead to sensory problems in foods. Peculiarly, the human olfactory system is extremely sensitive to TCA molecules. In the case of wine, TCA sensory threshold levels are often about 1.4–1.5 ng/L (**Table 1**) or lower (especially for white or sparkling wines) and typically vary up to 3–4 ng/L. Generally, the variations in sensory threshold values occur due to the following factors:

Wine matrix. First, the ethanol content in wine increases TCA threshold levels (in comparison, TCA sensory thresholds in water are much lower, starting from about 0.03 ng/L [16]). Second, the overall wine aroma intensity has a masking effect on the TCA perception. Therefore, TCA sensory thresholds are higher for wines made from aromatic grape varieties. In addition, TCA is usually better masked in red wines, as their aroma composition is often more intense compared with white wines. Woody notes in wine can also mask TCA defects, especially in the case of white wines [7].

Personal characteristics of tasters. The sensitivity of people to TCA can vary significantly depending on their olfactory system particularities, the current physiological state of sense organs [17], as well as their experience and training. Thus, the knowledge of "*cork taint*" has been found to be negatively correlated with individual TCA detection thresholds, i.e., awareness about *cork taint* increases the sensitivity of tasters to TCA [10].

Mode of sensory evaluation. Comparison of orthonasal (smell) and retronasal (volatiles traveling from the mouth into the nasal cavity) approaches shows that the latter usually provides a higher sensitivity to TCA. This effect is explained by the increased volatility of aroma substances at higher temperatures in the mouth. Another aspect of sensory evaluation is related to the tasters' attitude toward the perceived TCA smell. For example, it was shown that wine consumers could detect TCA at a concentration of 2.1 ng/L in the wine (detection threshold) and tolerate it, while for the consumer rejection threshold, the TCA content had to reach the level of 3.1 ng/L [10].

Fatigue and suppression of olfactory receptors. Already after a short exposure of tasters to *cork tainted* wines, their sensitivity to TCA drops rapidly and significantly (fatigue/adaptation effects). The mechanism of TCA interaction with olfactory system is not thoroughly studied. Nevertheless, TCA has been shown to attenuate olfactory transduction, which can lead to the suppression of wine aromas in general [18]. Moreover, such suppression was observed even at extremely low TCA concentrations, which are below the defined sensory thresholds. The masking of certain

wine notes by infra-threshold TCA concentrations (0.1–1 ng/L) was demonstrated for various wines [19–21].

2. Origin and precursors of TCA in cork material and wine

In order to work on preventive measures against TCA defects, it is important to identify its origin in cork and wine. The presence of hyperhalogenated molecules such as TCA in nature is associated with anthropogenic activities. Precursors of haloanisoles (including TCA) are halophenols (PCP—pentachlorophenol, TCP—2,4,6-trichlorophenol, etc), which for many decades in the twentieth century were widely used as components of chlorophenol-based biocides: herbicides, insecticides, fungicides. These products were extensively utilized in agriculture, for the treatment of wooden materials, cardboard, textiles, etc. [22, 23]. Since that time, the problem of *cork tainted* wines began to attract more and more attention, as mentioned at the beginning of the review. PCP, TCP, and other chlorophenols are relatively stable molecules, but hyperchlorinated phenols can slowly degrade, losing chlorine atoms in the structure (e.g., PCP → TCP). As a result, these compounds can spread and persist in ecosystems for decades and accumulate in cork trees or soil, serving as one of the possible precursors of TCA in cork (**Figure 1**) [22, 23]. Once TCP is in the bark or wood, the formation of TCA occurs microbiologically, which involves *O*-methylation of TCP (**Figure 2**). *Penicillium*, *Fusarium,* and *Trichoderma* strains are considered as microorganisms, which are able to carry out this bioconversion at high and moderate levels [15, 24]. The physiological reason for biomethylation by these filamentous fungi is a defensive response to TCP, which acts as a strong toxin (fungicide). Filamentous fungi are widely spread in nature and do not produce TCA without its precursor TCP. Therefore, the objective reason for the formation of TCA in cork and wood is not the presence of filamentous fungi, but the contamination of these materials with cholorphenols, in particular TCP.

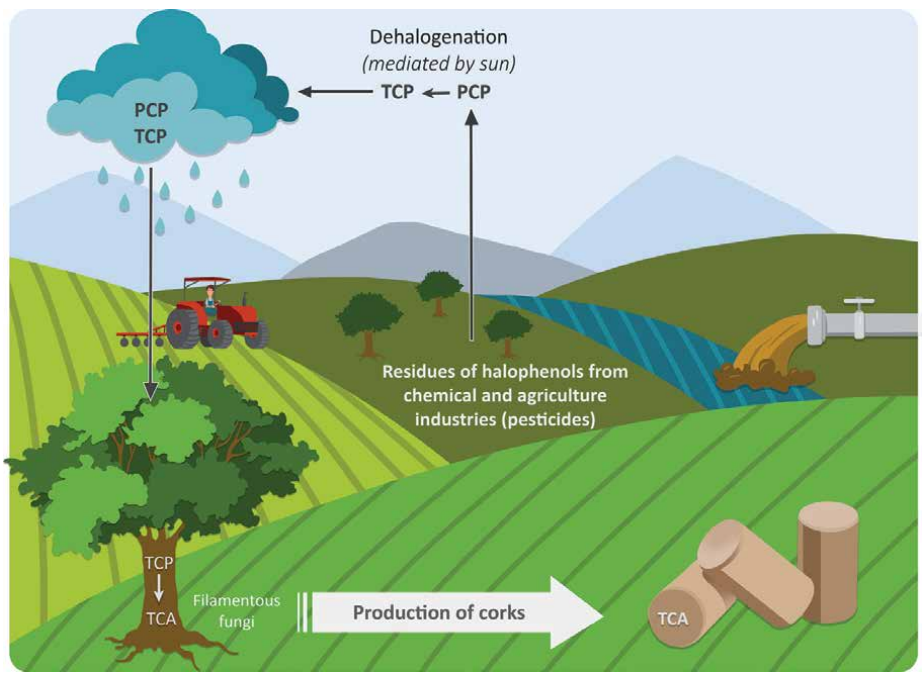

Figure 1.
Possible pathways of environmental contamination of cork trees with TCP and TCA (based on [22]).

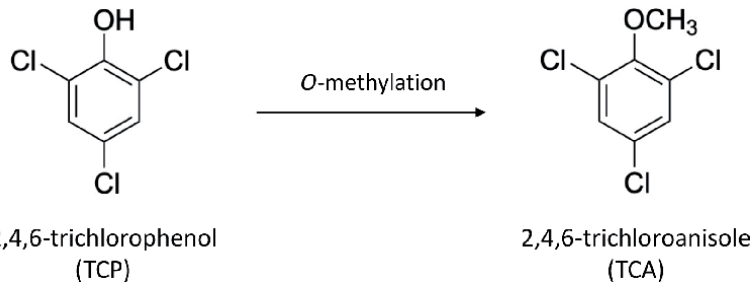

Figure 2.
Microbiological formation of TCA by O-methylation of TCP.

Besides the fact that TCP and other chlorophenols are banned as biocides in many countries, these compounds can still be found in many places in nature. The latter also include remote areas that have not been directly treated with these biocides, but contaminated by waterways and atmospheric precipitations (**Figure 2**) [25]. In general, a limited number of organisms are capable of transforming halophenols, which results in a low degradation rate of these compounds in nature. In addition, there are also other pathways of TCP accumulation in cork and wooden materials, which are described in the following subsections.

2.1 Origins of TCA in cork stoppers

Exogenous contamination of trees by biocides represents the important origin of TCP in bark and wood. As discussed above, TCP is microbiologically transformed into TCA, the latter accumulates in bark, from which cork stoppers are then produced. However, there are also other sources of TCP and TCA in the cork material, some of which were quite relevant in the past. One of these pathways of TCA formation starts from the *chlorination of phenol* present in cork. Phenol is formed in cork and wooden materials by degradation of lignin and by the action of *Penicillium* spp. These fungi are able to synthesize phenol starting from glucose following the pentosephosphate and shikimic acid pathways [26]. Then the treatment of cork with chlorine-containing agents can lead to the chlorination of phenol yielding various chlorophenols, including TCP and dichlorophenols (**Figure 3**). Such cork treatment was widespread before 1990 during the production process of corks:

- bleaching of cork cylinders with calcium hypochlorite solution $Ca(ClO)_2$;
- boiling of bark slabs with tap water containing chlorine Cl_2.

Chlorination of phenol is a chemical process, however, some authors suggested that biochemical transformation by *Basidiomycetes* can also take place under certain conditions [23]. As was already discussed, the formation of TCA involves the *O*-methylation step, which can occur before or after chlorination of phenol (**Figure 3**). One of the signs of the use of chlorine-containing substances in the manufacture of corks is the presence of other compounds, such as chlorocresols and chloromethylanisoles, which have a moldy off-odor similar to TCA.

Nowadays, in order to protect the quality of cork stoppers, the application of chlorine-based treatments is strongly discouraged by the "International Code of Cork Stopper Manufacturing Practices" promoted by the European Confederation of Cork (C.E. Liège) [7]. The practice of hypochlorite usage as bleaching agent was banned around 1990 and completely abandoned by all cork stopper producers.

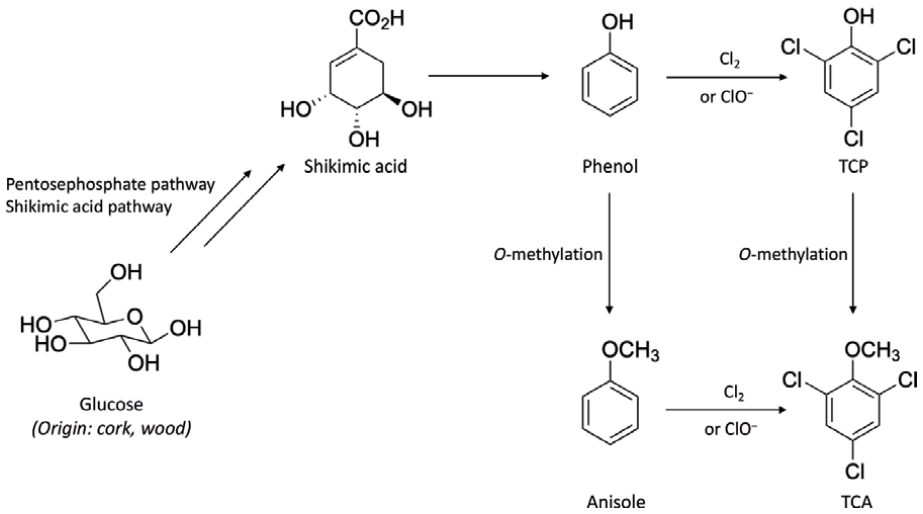

Figure 3.
TCA formation via *chlorination of phenol in cork and wooden materials (based on [26, 27]).*

Hypochlorite was substituted by hydrogen peroxide H_2O_2 that does not cause haloanisole problems. The application of chlorine-containing tap water for the bark slabs boiling process is also forbidden. As a result of these measures, along with the improved analytical control, the average cork contamination was significantly reduced, but the TCA problem was not completely resolved.

The other potential source of TCP in cork material is *degradation of PCP*. Among chloroanisole-based biocides, PCP was probably the most utilized. Thus, in the 1970s in the United States alone, its production reached about 23 k tons per year [28]. Unsurprisingly, PCP is still abundant in nature and in wooden materials. In the presence of some bacteria, the reductive dechlorination of PCP occurs as a part of the chlorophenol degradation process (**Figure 4**), which implies replacement of chlorine atoms by hydrogen and formation of less chlorinated phenols.

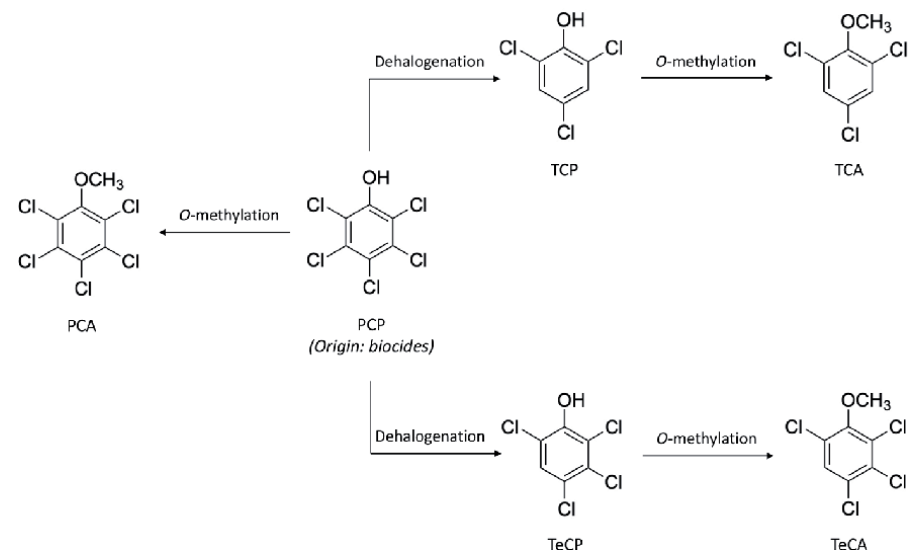

Figure 4.
Dechlorination reactions of PCP and formation of chloroanisoles.

Compound	Threshold levels	References
2,4,6-Trichloroanisole (TCA)	from 1.4–1.5 ng/l	see **Table 1**
2,3,4,6-Tetrachloroanisole (TeCA)	5–15 ng/l	[31]
Pentachloroanisole (PCA)	> 50 µg/l	[31]
2,4,6-Tribromoanisole (TBA)	3.4 ng/l	[31]

Table 2.
Sensory thresholds of haloanisoles in alcoholic solutions (wine).

Among others, TCP and 2,3,4,6-tetrachlorophenol (TeCP) can be observed as products of dehalogenation [25, 29, 30]. All these chlorophenols can be microbiologically converted to corresponding chloroanisoles: TCA, TeCA, and PCA. The concentration of the latter in wine can be even higher than TCA, however, PCA does not play a prominent role in *cork taint*, since its sensory threshold is higher by 3–4 orders of magnitude and is measured in µg/L (**Table 2**).

Given the different origins of TCA in cork stoppers, it is sometimes unclear which pathway contributes to the formation of TCA in each specific case. The cork stoppers production process (**Figure 5**) includes steps, which are aimed at reducing the TCA content originating from contaminated trees. Among these processes are the aeration of bark slabs, extraction of contaminants by boiling of bark slabs in water, etc. However, all these efforts to reduce TCA may be futile if the succeeding production steps are poorly controlled. For example, TCA can be subsequently regenerated in the treated cork material if the bark slabs are stored and transported wet. Under these conditions, fungi develop rapidly and biomethylation of TCP leads to reappearance of TCA. Therefore, it is necessary to strictly monitor all critical stages in the cork stopper production. Over the past decades, many efforts and technological improvements have been implemented by cork producers to reduce

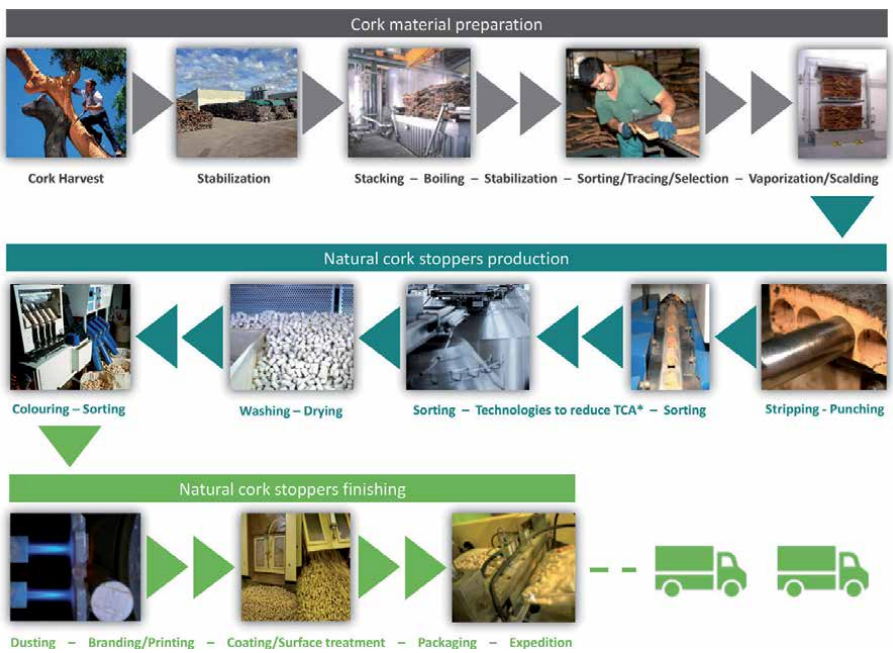

Figure 5.
*Typical steps in the production of natural cork stoppers (*more details in section 4).*

and control the fungi growth, prevent the TCA formation in cork material and its removal during the production process.

Finally, if contaminated corks are detected, the origin of TCA can be deduced from the simultaneous analysis of haloanisoles, halophenols, and their ratio. For example, the presence of dichlorophenols in cork or tainted wine indicates the probable involvement of chlorine at some stages of cork stopper production (**Figure 3**) rather than TCP precursor from the forest [23].

2.2 Other sources of TCA in wine

Musty/moldy defects in wine caused by TCA cannot be attributed only to cork stoppers. There are cases when wines are bottled with plastic closures or screw caps and can still be contaminated with TCA. These incidents have happened in the past and continue to surprise wine producers and consumers today. Possible ways of such contamination are as follows:

- *Contaminated air and winery equipment*. Formation of haloanisoles, including TCA, is possible directly in wine cellars. Corresponding precursors, TCP and other chlorophenols, can be present in various wooden elements: roof constructions, walls, floor, paints, pallets, barrels, etc. [32, 33]. These precursors often originate from chlorophenol-based biocides, which were used in the past as fungicides for wood protection or paint preservatives, or are formed from the reactions of chlorine-containing detergents with wood components in the cellar, as shown in **Figure 3**. Then, filamentous fungi produce TCA (**Figure 2**), which is volatile and contaminates the air. Subsequently, TCA can be easily absorbed by winery equipment, plastic hoses, filter sheets, bentonite, wooden barrels, various enological products, and transmitted to the wine once it gets in contact with the contaminated surfaces (**Figure 6**). The described scheme of wine contamination is more typical for old cellars, where wooden constructions, paints, plasters, walls can contain remarkable quantities of chloroanisole precursors. Nowadays, these compounds are forbidden as biocides, however, other risks of air contamination also exist in modern cellars. Bromophenol-based biocides (2,4,6-tribromophenol, TBP) are still allowed for the wood treatment and can be present in paints, resin laminates, etc. [34]. Similar to the reaction in **Figure 2**, filamentous fungi are able to convert TBP to 2,4,6-tribromoanisole (TBA), which has analogous sensory properties as TCA: musty/moldy off-odor and low sensory perception threshold (**Table 2**). Therefore, the current analysis of musty/moldy wines usually includes the determination of not only TCA and chloroanisoles, but also TBA. Once the source of TCA or TBA in the cellar is identified, it should be eliminated. If it is not possible and the air contamination is not very high, then intensive air ventilation may be the solution. Among the preventive measures is the replacement of wooden elements in the cellar, e.g., metallic or plastic pallets instead of wooden ones. The utilization of chlorine-containing detergents to clean the winery and equipment should be avoided. Finally, it is recommended to periodically check the air in the cellar for various contaminants. The standardized method of halophenols and haloanisoles analysis in air involves passive sampling by bentonite spread out over a strip of aluminum foil and exposed to the atmosphere for at least 5 days [35]. Then the contaminants are extracted by ether/hexane mixture (or other solvents) and analyzed by GC–MS. Active sampling methods were also suggested, e.g., pumping air through the tubes with Tenax TA™ sorbent followed by thermal desorption – GC – triple quadrupole MS [36].

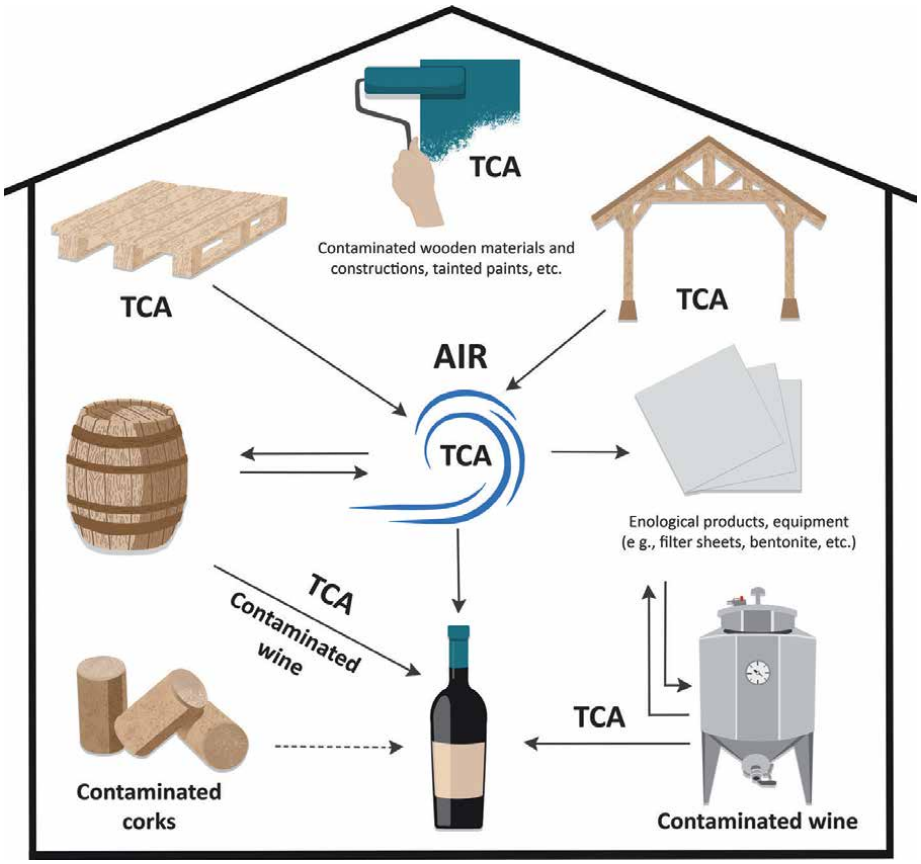

Figure 6.
Possible ways of wine contamination with TCA in a cellar.

- *Secondary contamination of wine closures.* Besides contaminated winery equipment, wine closures can also accumulate and transmit airborne TCA. Cork and plastic materials of various wine closures have a great ability to absorb TCA. Thus, even a short-term exposure of cork stoppers to a contaminated atmosphere (24 hours) is sufficient to intake a large amount of TCA [37]. The main part of absorbed TCA is initially localized in the outer 2 mm of the cork cylinder. Then it migrates inside the closure, most likely along the lenticels. As for plastic closures, the absorption of TCA is also significant, and migration inside these closures is more efficient, since they do not have a cellular structure like natural corks. An example of such a way of contamination was reported already in 1990 [38]. After transporting champagne corks to Australia, the stoppers were found to have a TCA pollution. The corks were packed in polyethylene bags inside fiberboard cartons, which contained significant amount of TCA. Investigation of the materials that came to contact with the packaging suggested that the source of TCA was the floor of the shipping container, which was treated with fungicides containing TCP. In addition, Schaefer presented a number of examples of TCA contamination [39], e.g., pollution of screw caps (liners) that were stored in cardboard boxes on contaminated wooden pallets. In particularly, a higher TCA content was observed in the screw caps, which were on the bottom of the box.

- *Contamination through wine closures after bottling.* In the early 2000s, research began on the possibility of TCA migration from the air through bottle closures

into wine. Several studies demonstrated that different grades of natural and agglomerated corks are excellent barriers against airborne d_5-TCA for at least 2–3 years of bottle storage in a contaminated atmosphere [40–43]. The analysis of these stoppers revealed that d_5-TCA was detected only on the top of the closures, which was in contact with the contaminated air. As for other types of closures, certain amounts of airborne d_5-TCA were found in wines sealed with some types of synthetic stoppers, glass stoppers, and screw caps (excluding those with Tin Saran liner). One of the possibilities to protect wines with plastic stoppers from the airborne haloanisoles contamination is to use capsules without holes. This approach allowed to reduce the wine contamination with airborne d_5-TCA by about 10 times or more [44]. A possible criticism of many of these studies about the migration of TCA through bottle closures is that the applied storage conditions involved relatively high levels of air pollution. At the same time, there are no comprehensive reviews summarizing the TCA levels in air in real polluted environments. As for real cases of wine contamination *via* this mechanism, one of them was described in the Annual Report of Australian Wine Research Institute [45]. A large batch of sparkling wine with crown seals (about 14 months after *tirage*) was analyzed because of the musty taint, and the presence of TeCA and traces of PCA was determined. As a result of the investigation, it was suggested that several months of exposure to the contaminated air allowed the migration of TeCA through the crown seals in quantities sufficient to taint the wine. Wood preservatives were identified as a potential source of haloanisoles.

Given all of these potential pathways for TCA contamination, there is a need to more comprehensively investigate the problems associated with musty/moldy wines rather than simply linking them to cork stoppers.

3. Methods of TCA analysis in cork stoppers

Cork stoppers may eventually contain at least traces of haloanisoles, in particular TCA. However, wines bottled with cork stoppers only rarely have noticeable musty/moldy defects. The reason lies in the particularities of the extraction of TCA by wine from the cork material. Wine is an aqueous solution of alcohol with a moderate extraction power in relation to TCA, while the cork material retains this compound rather strongly [46]. In addition, TCA can be efficiently extracted only from the part of the cork that is in direct contact with wine. No noticeable migration of TCA from the middle or outer part of cork stoppers into bottled wine is usually observed [40]. Consequently, the amount of TCA extracted by wine is far from the entire TCA content inside corks. According to different authors, the part of TCA that can be released into wine from a cork stopper typically varies between 0.05% and 8% [3, 33, 47, 48]. Considering these peculiarities, two concepts of TCA contamination of cork stoppers were introduced:

- *Releasable TCA*, which is defined as the equilibrium value of TCA that a given cork imparts to the soak solution (wine) and is measured in ng per liter [48];

- *Total TCA* corresponds to the entire content of TCA in a cork stopper and is expressed in ng per gram of cork material.

In general, *releasable TCA* depends on *total TCA* content and its localization in the cork stopper. Determination of *releasable TCA* content has an extensive practical

application. Namely, it corresponds to the amount of TCA, which can potentially migrate and contaminate bottled wine. Therefore, it became a routine technique to control *releasable TCA* content in cork stoppers at different stages of their production. On the contrary, *total TCA* analysis most often serves as an important tool for scientific purposes. It allows to study the nature and origin of cork contamination, the distribution of TCA inside corks [49], the dynamics of TCA absorption by cork material from wine [46] or from the air [37], etc. For example, it was found that TCA content in the lenticel and non-lenticel cork fractions did not differ considerably, as well as TCA concentration in the light and dark parts of the growth rings [49]. Analytical approaches to determining *releasable TCA* and *total TCA contents* are comprehensively discussed in our review [50], including the particularities of the described methods: sample preparation and treatment techniques, TCA recovery, detection of other analytes (haloanisoles and halophenols), etc. In the current book chapter, this information is summarized in the following subsections.

3.1 Analysis of releasable TCA content

Releasable TCA values may vary depending on the cork soaking conditions: alcoholic strength of extractant, time of maceration, etc. In order to overcome these uncertainties, standardized procedures were developed. Two analytical methods proposed by OIV organization (Method OIV-MA-AS315–16 [51]) and ISO (20752:2014(E) [52]) are currently in wide use. According to these protocols, cork stoppers are macerated in an aqueous-alcoholic solution (12% vol. alcoholic strength) or white wine (10–12% vol. [51]) during 24 ± 2 h of passive soak. This time is sufficient to ensure the equilibrium for TCA extraction when it reaches a steady state [53]. Additional studies have shown that maceration time can be reduced by using active soak, for example, up to 2 hours with microwave assisted extraction (MAE) [54]. The MAE technique provides results very similar to the standard soak procedure for corks with *releasable TCA* < 25 ng/L. Once obtained, extracts are usually analyzed by GC–MS or GC-ECD in combination with headspace solid-phase microextraction (HS-SPME) [51, 52] or stir bar sorptive extraction (SBSE) [54].

The soaking of cork stoppers can be done individually or in groups. The latter approach is commonly used on an industrial scale for quality control of commercial batches of cork stoppers. Overall, comparable results have been found for group soak values and average values of individual cork soaks (R^2 about 90%) [48, 53]. The size of glass containers and the volume of extractant for *releasable TCA* analysis usually depend on the number of corks. For example, group extractions of 20 and 50 corks are recommended to be done in 1 L and 2 L containers, respectively [51, 52]. There are no exact recommendations regarding the volume of extractant, but the cork stoppers should be completely immersed in the solution. It has been demonstrated that a reasonable deviation of the extractant volume does not significantly affect the TCA equilibrium and the resulting *releasable TCA* values [48]. Further studies of the adsorption/desorption process of TCA on the cork surface revealed certain limitations of the method. For example, a group soak can demonstrate an undetectable level of TCA even though some individual corks may release a certain amount of contaminant. This may occur because "clean" cork stoppers can reabsorb most of TCA from the group extract. Thus, in one study it was shown that cork stoppers are able to remove about 80% of TCA from contaminated wine after 24 h of soaking [46]. Therefore, individual soaking can be a more representative test compared with group soaking. At the same time, the results of individual soaking can also be distorted due to the reabsorption of TCA by "clean" parts of the same cork.

Despite the described adsorption/desorption effects, the values of *releasable TCA* analysis for individual stoppers correlated quite well with the TCA content in wines bottled with the same corks [48]. Thus, it was found that 14 months after bottling, on average, the concentration of TCA in wines was about half the corresponding *releasable TCA* values. The lower TCA content in real conditions can be due to the fact that the wine contacts only a limited surface of the cork in the bottle, while during the *releasable TCA* analysis, the entire cork is immersed in the extractant.

For the analysis of cork extracts, the same GC methods are used as for the analysis of wine [55, 56]. Therefore, in addition to TCA, other haloanisoles (TeCA, PCA, TBA, etc.) and halophenols can also be quantified. For a more accurate determination of the latter (TCP, TeCP and PCP), preliminary derivatization of extracts (acetylation) can be carried out [57]. Finally, in addition to GC methods, a bioanalytical technique for the analysis of wine and cork extracts (Bioelectric Recognition Assay (BERA)) was studied [58]. This technique is based on a biosensor containing membrane-engineered cells with inserted TCA-specific antibodies. Therefore, it is limited only to the TCA determination and operates in the range of about 1–12 ng/L. On the other hand, BERA is a relatively fast analysis, requiring only 3–5 min, and can be considered as a promising express method.

3.2 Analysis of total TCA content

The key concept of this method is the maximum extraction (recovery) of hydrophobic haloanisoles from the cork matrix. This can be achieved by selecting an effective solvent and grinding the cork to obtain a large surface in contact with the extractant. Corks can be ground in a granulating mill with a stainless steel bowl [59] or in a regular coffee grinder [46]. It is recommended to pre-freeze corks to facilitate the grinding process and prevent the loss of volatile organic compounds due to evaporation. Freezing can be done by immersing a cork stopper in liquid nitrogen [60, 61]. To increase the repeatability of the analysis, it is recommended to make the fraction of ground cork less than 3 mm [35] or even homogenize it by passing it through a sieve, e.g., 1 mm in diameter [61, 62]. At the same time, the analysis of pieces around 5 x 5 mm also demonstrated good recoveries and repeatability [63].

Among the tested solvents, hexane and pentane showed high extractive properties with respect to hydrophobic haloanisoles and are now widely used [2, 63, 64]. According to the OIV protocol, an ethyl ether/hexane mixture (50/50; v/v) is recommended [35]. Alcoholic solutions with an ethanol concentration of more than 50% (vol.) showed lower but still good results. In particular, a solution with 75% (vol.) of ethanol can be recommended in certain situations, for example, in the case of a subsequent SBSE analysis technique [59]. Methanol in combination with some extraction methods is also a good candidate for analysis [62]. Other solvent options have also been described, but they are not widely used or are specified for certain extraction methods: pentane/ethyl acetate [4], pentane/diethyl ether for pressurized liquid extraction (PLE) method [60], etc.

With regard to extraction techniques, there are several approaches that include conventional soak, Soxhlet extraction, and various advanced methods. Conventional soak of ground cork is usually performed in closed glass vessels, and variations are related to the selection of solvent, extraction time, application of mechanical agitation, etc. Generally, the method is effective, but time-consuming: typically maceration takes 24 hours without mechanical agitation [37, 46, 63, 65]. Maceration time can be significantly reduced by using agitation in a rotary mixer

[64] or vortex [35], by sonication in an ultrasonic bath (15–30 min) [59, 64] or immersing an ultrasonic processor inside the cork/solvent mixture for 1–2 min [66, 67]. Conventional soak is an effective method with the possibility to achieve TCA recoveries of more than 90% [50].

Soxhlet apparatus provides continuous circulation of a boiling extractant through a ground cork. Extraction time usually varies between 7 and 24 hours [33, 62, 68], making this method not time-efficient. Nowadays, Soxhlet extraction is less often used as a routine technique, but remains a reliable reference method due to its high TCA recovery (up to 99%), repeatability, reproducibility, and small deviation between replicates [62, 64].

Both conventional soak and Soxhlet extraction result in a relatively large amount of extract, which must be concentrated prior to injection for GC analysis. Therefore, the improvement of extraction methods was aimed not only at optimizing the time, but also at reducing the volume of solvent used. It has been proposed to utilize the following special extraction techniques for haloanisoles: microwave-assisted extraction—MAE [62], supercritical fluid extraction—SFE [69], pressurized liquid extraction—PLE [60], pressurized fluid extraction—PFE [70], etc. All of these advanced extraction methods demonstrated excellent efficiency (high recoveries and good reproducibility), but they require specific equipment.

The next steps in the development of cork analysis are organic solvent-free methods, which involve heating ground cork with or without water. As a result, TCA and other haloanisoles are vaporized and then analyzed, for example, using HS-SPME [61, 71]. These methods of direct analysis do not require special sample preparations, but are carried out with a smaller amount of analyzed cork, e.g., 200 mg or less. A similar approach was also proposed for the analysis of entire natural corks and is discussed in Section 4.2.2.

Finally, the determination of *total TCA* and other haloanisoles and halophenols can be performed not only for cork stoppers, but also for various objects present in cellars: wooden pallets [33], oak barrel sawdust [72, 73], wooden chips [35], wooden staves [74], and other cellar materials [39]. All of these materials should be preliminary ground, as it is required for corks [35].

4. Strategies to avoid TCA presence in wine

It is more practical to prevent TCA contamination of wines during their production, bottling, and storage process than to remove the *cork taint* later. Strategies for avoiding haloanisoles pollution of the winery environment, equipment, enological products are well described by Jung and Schaefer [27] and are partially mentioned in *Section 2.2* of this chapter. The current section will discuss how to reduce/eliminate TCA contamination in cork stoppers. Being among the most unpleasant and most frequent wine defects, the *cork taint* problem has triggered numerous research projects led by cork industry players over the last 30 years to remedy this situation.

One group of the early methods was aimed at sterilization of cork material (to eliminate microorganisms producing TCA) and decontamination. The corresponding technologies involved exposure of cork material to microwave radiation [75]; treatment of cork with alkaline solutions [76, 77], etc. Other methods were focused on the elimination of chlorophenols (TCA precursors) from the cork material, such as treatment of cork with a phenol oxidizing enzyme [78] or application of *Chrysonilia sitophila* fungi, which are able to degrade TCP without formation of TCA and inhibit growth of TCA-producing fungi [79].

The use of physical barriers on a cork stopper to prevent it from making contact with bottled wine is another strategy that has been tested. For example, a silicon joint

on champagne stoppers was studied to prevent the migration of TCA into wine [80]. Another study investigated a nanostructured carbon-based film on the cork surface as a barrier against dust and impurities that may penetrate and pollute the cork mass [81].

Most of the approaches listed above demonstrated only limited effectiveness in diminishing TCA in cork stoppers and its appearance in bottled wine. Therefore, there are currently two main strategies for dealing with contaminated corks:

- cleaning of cork material to remove TCA;
- sorting of cork stoppers to select "TCA-free" ones.

More details on these strategies, their limitations, and associated difficulties are presented in the following subsections.

4.1 Technical methods to reduce/eliminate TCA presence in cork stoppers

Various products and techniques were proposed for cleaning and eliminating TCA from contaminated corks: for example, treatment with an aqueous suspension of activated charcoal [82] or a mixture of water and organic solvents (including ethanol) combined with a heating phase obtained with electromagnetic energy at hyper frequencies [83], etc. Not all tested cork cleaning methods have shown high efficiency, reasonable installation costs, processing and energy consumption, as well as safety requirements. In addition, some processes can have secondary effects that cause significant changes in the physical and chemical composition of corks, leading to the alteration of their mechanical or sensory properties. As a result, there are a limited number of cork cleaning technologies that have proven their practical applicability and suitable for use on an industrial scale. Among these approaches are treatment with steam, thermal desorption by vacuum, and treatment with supercritical CO_2.

4.1.1 Treatment with steam

It is known that the concentration of TCA in cork can be diminished by simple aeration, which can be accelerated by higher temperature and humidity [37, 84]. Therefore, steam distillation technique was proposed to remove volatile substances, including TCA.

Steam extraction technologies are used nowadays by different cork manufacturers and demonstrate good results (**Figure 7**). For example, the first industrial steam cleaning process ROSA® of Amorim Cork provided the removal of about 80% of TCA from cork granules [86], which are then used to produce agglomerated cork stoppers. Subsequent optimization of the process led to a reduction in the TCA content to almost "zero" level (i.e., below the limit of quantification (LOQ)) for cork granules, which possessed the initial *releasable TCA* levels less than 6 ng/L. The next development step allows treating entire natural cork stoppers (ROSA Evolution®), reducing their *releasable TCA* levels by 80–85%.

Other companies that use steam to clean natural corks and granules also have their own particularities in the process (Innocork® and Vapex®, by Cork Supply; Neotech® and Sara Advanced®, by M.A.Silva; Revtech and others). For example, utilization of an ethanol-water vapor mixture to treat corks (Innocork®). The process can take place under 60°C allowing reduction of the TCA content up to 80% [87]. Higher temperatures above 70–80°C are not recommended, because they led to irreversible distortions of the stoppers after cooling [87]. Atmospheric pressure

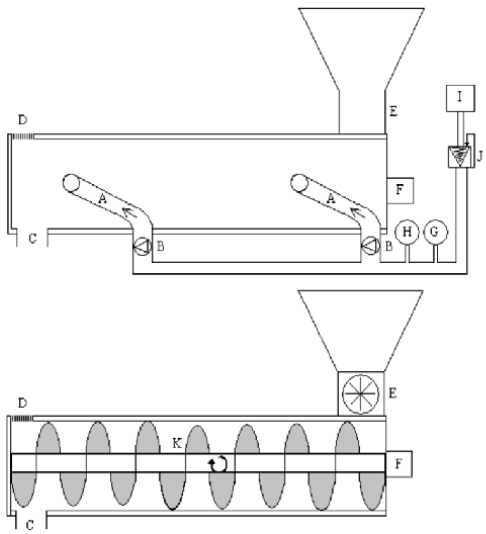

Figure 7.
Steam extraction technology (ROSA®) for TCA extraction from cork granules [85].

is suitable for these cleaning technologies as it provides good extraction results at a considerable cost reduction (no special low-pressure equipment is required). At the same time, a higher or lower pressure (0.2–0.8 bars) or a variation of pressure in the cleaning system can be applied to increase the efficiency of TCA removal. For example, Belighit with colleagues (2010) proposed cycles of pressurization with water vapor followed by periods of vacuum to enhance the cork cleaning [88].

4.1.2 Thermal desorption by vacuum

Removal of TCA and other compounds by thermal desorption involves increased temperature to enhance the volatilization of contaminants from the cork material, which is facilitated by vacuum [89]. The desorption process requires temperatures above the boiling points of the haloanisoles to convert the contaminants to a gaseous state. This temperature for TCA at atmospheric pressure (1 bar) is about 240°C, which can compromise the composition of cork material. At the same time, boiling points can be substantially decreased by applying a vacuum: for example, 0.1 mbar pressure lowers the boiling point of TCA to 19.5°C. The desorption process can be carried out at a deeper vacuum of 0.01 mbar or lower, which further facilitates the volatilization of TCA. As a result, desorption of TCA and other contaminants can be performed at moderate temperatures if the proper vacuum level is applied [90]. In addition, the preliminary "recrystallization" of TCA (boiling corks in water and subsequent drying) before the thermal desorption process allegedly enhances the removal of pollutant [91]. A recent example of industrial application of thermal desorption processes is Naturity® technology (Amorim Cork), which allows the extraction of TCA and similar compounds from natural cork stoppers with high efficiency.

4.1.3 Treatment with supercritical CO_2

Supercritical fluid is a special state of matter, which exists at elevated temperature and pressure above its critical point and beyond the distinct liquid and gas phases. For carbon dioxide (CO_2), this supercritical phase can be reached by subjecting it to pressures over 73 bar and temperatures over 31°C (**Figure 8**). Under these conditions, CO_2 is neither liquid nor gaseous, but combines the properties of both states. Its "gaseous"

properties give CO_2 a very high diffusion capacity through a treated material (e.g., cork), while its "liquid" behavior provides a very high extraction power toward some volatile molecules (e.g., volatile and malodorous compounds of cork, including TCA). By adjusting the pressure/temperature conditions (e.g., 120 bars/60°C), it is possible to optimize the extraction of TCA from cork by CO_2 while preserving the mechanical

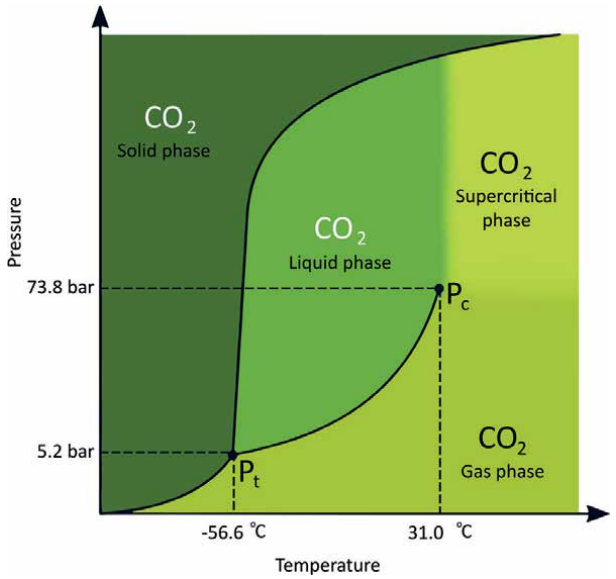

Figure 8.
Pressure–temperature phase diagram for CO_2.

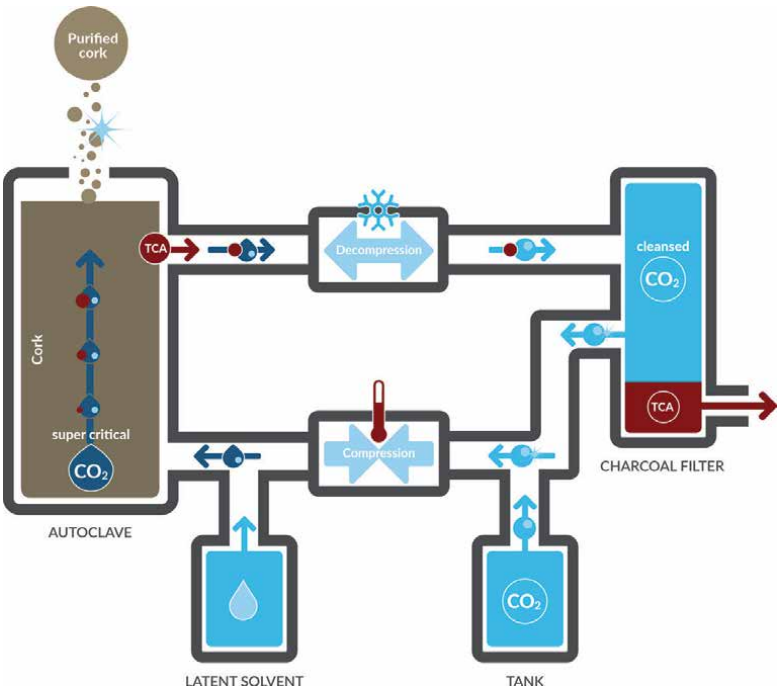

Figure 9.
Supercritical CO_2 cleaning technology (Diamant®) to remove TCA and other volatile compounds from cork granules.

properties of the cork material [92]. This extraction process does not require the use of organic solvents, which makes it safe for human health and environmentally friendly.

Diamant® (Diam Bouchage) was the first industrial system for supercritical CO_2 cleaning of cork material based on a technology patented over 20 years ago (**Figure 9**) [93]. It has been shown that this cleaning process is highly effective in achieving "zero" levels of residual TCA (i.e., below LOQ = 0.3 ng/L) in a single treatment cycle of cork granules, which had an initial contamination close to 20 ng/L of *releasable TCA* [92], and later close to 50 ng/L [94]. In addition, over 150 other molecules besides TCA are also removed (mainly nonpolar), including various terpenes, pyrazines, etc. [95, 96]. Further development of supercritical CO_2 extraction technology for cork material involved optimization of the used energy and the CO_2 volume. A recent example of other technologies based on the similar principle is Xpür® (Amorim Cork), which was also designed to clean cork granules.

Generally, the existing supercritical CO_2 extraction technologies for cork material are limited to cork granules, which are subsequently used to produce agglomerated stoppers. Applying this process to natural cork stoppers encountered certain difficulties. The process efficacy was greatly reduced due to the low diffusion of supercritical CO_2 in the cork structure: when growing on a tree, the cork acquires a nonisotropic internal structure, i.e., its physical and mechanical properties (elasticity) are not the same and depend on the orientation of the cork growth lines. During the supercritical CO_2 cleaning process involving pressurization and decompression, the cork compresses and then decompresses unevenly, generating fractures in the material. This results in delamination of cork growth veins, a loss of its physical properties of about 30%, and a significantly increased heterogeneity of oxygen permeability levels among cleaned natural cork stoppers. In turn, micro-agglomerated cork stoppers made of cork granules provide far superior homogeneity and consistency.

Supercritical CO_2 extraction was proposed also for the determination of *total TCA* in ground corks [69]. In addition, this technology is widely used nowadays in other industries, as it allows the treatment of raw materials at moderate temperatures avoiding side processes (e.g., Maillard reactions) and the formation of undesirable by-products. Thus, it is commonly used in perfumery to extract aromatic molecules from natural materials, in the food industry to extract caffeine from coffee (producing decaffeinated coffee), theine from tea, lupulin from hops, etc.

4.2 Quality control techniques: selection of "TCA-free" cork stoppers

As it was mentioned in Section 3, the analysis of *releasable TCA* is used for the quality control of cork batches. The corks are randomly selected, analyzed, and the results are extrapolated to the entire batch of stoppers. Therefore, a purchaser of these natural corks can count on the probability of contamination within the batch, but not on the specific TCA contamination of each individual cork. To guarantee the "TCA-free" status of each stopper, they need to be analyzed individually, one by one. The usual *releasable TCA* method is not suitable for this goal and is considered destructive: soaking and following drying procedures alter the cork surface due to tannin staining [97] and other effects. Therefore, the aim was to develop non-destructive methods, which could correlate with the *releasable TCA* analysis. As a result, "TCA-free" corks can be selected from the analyzed batch, commercialized, and used later for wine bottling. Nowadays, there are two main nondestructive approaches to the individual cork analysis, which will be discussed below: sensory methods and automated methods.

4.2.1 Sensory methods

The high interest in the sensory evaluation of corks in the late 1980s and 1990s led to the development of the first protocols for analysis of stoppers [98, 99]. In 1996, a typical sensory method of cork analysis was elaborated at the Hochschule Geisenheim University (former Forschungsanstalt Geisenheim), which according to the latest issue [100] offers the following procedure:

- 3 ml of water is added to a 100 ml glass flask and a cork stopper is placed inside;
- the flask is closed and stored at room temperature for 24 hours (to achieve equilibrium of the volatile compounds of the cork in the vapor phase);
- sensory evaluation of the air from the vials by sniffing by trained tasters.

Other routine sensory evaluation methods of cork stoppers can vary somewhat in terms of flask volume, amount of water added, etc. [97, 101]. For example, Macku and colleagues [97] used 125 mL flasks with six drops of water. At the same time, the principles described above remain the same and are often referred to as "dry soak" sensory screening methods. The advantage of the sensory method also lies in the possibility to identify various aroma deviations related not only to TCA and haloanisoles. Among other off-odor compounds are geosmin, 2-methoxy-3,5-dimethylpyrazine, and various malodorous molecules, including those formed due to improper treatment of cork material during the production process.

To prove the effectiveness of the "dry soak" method, Macku and colleagues [97] performed an extensive sensory evaluation of 2000 corks. As a result, about 6% of the stoppers were rejected and then analyzed by GC–MS. About one-third of the rejected corks possessed *releasable TCA* levels above 1 ng/L, while the rest had levels below 1 ng/L (their discard can be related to the presence of other taint substances in cork). In turn, 100 stoppers from the "clean" group were randomly selected and also analyzed by GC–MS. None of these stoppers demonstrated a *releasable TCA* level higher than 1 ng/l, which is usually under the human perception threshold.

The "dry soak" method can be used for sensory screening of corks on an industrial scale. For example, the company Cork Supply adopted this technique for their natural corks, and selected "cork taint-free" stoppers became available to customers. Despite the proven effectiveness of the method, it is a time-consuming technique based on human factors, which can only be applied to a limited number of corks over a given period of time. Therefore, the market was waiting for automated methods of cork stoppers selection.

4.2.2 Automated methods

The purpose of automated methods is to quickly analyze each individual cork stopper for TCA content and then separate the corks into different groups depending on the TCA contamination. The general technical principle for cork analysis is as follows: a cork stopper is placed into a small hermetic chamber and heated, which induces vaporization of TCA from the cork; then the air from the chamber is collected and analyzed by GC–MS method with various detection systems [electron capture detector (ECD), ion mobility spectrometry (IMS), etc.].

Several companies have recently been developing such automated nondestructive technologies for the analysis of individual corks. The first system based on this principle, which started to work on an industrial scale, was NDTech® (Amorim Cork). Optimization of the technology allowed reduction of the time of analysis of one cork

to 15 seconds and provide the *releasable TCA* detection level of 0.5 ng/L. Thus, all analyzed cork stoppers with TCA levels below 0.5 ng/L are selected as "TCA-free" corks. Among other automated systems present on the market or in the commercial phase are the following: the system of CEVAQOE laboratory; Vocus Cork Analyzer (Tofwerk); the system of Cork Supply Portugal, S. A. (cork company); the system developed in collaboration between Bruker (scientific instruments manufacturer) and Egitron.

Automated systems for the analysis of TCA in corks are more efficient than sensory methods. However, considering the cork market, which requires billions of stoppers per year, even the automated methods available cannot analyze all the corks produced. Therefore, these technologies remain focused rather on higher-quality corks for wines in the medium- and high-price segments.

5. Removal of TCA from contaminated wines

Approaches to remove TCA from contaminated wines have been developed over several decades. Haloanisoles are nonpolar compounds; therefore, various hydrophobic materials (including different polymers) have been tested as candidates for diminishing TCA content in tainted wines (**Table 3**). Polyethylene, as a widespread and inexpensive plastic material, has shown high scalping properties in relation to TCA. It has been used in the form of a film [46] or granules (ultrahigh-molecular-weight polyethylene (UHMW PE). In general, polyethylene is able to absorb more than 90% of TCA [46, 102] and other haloanisoles from wine [46]. The efficiency of immersed film treatment depended on the film thickness, contact surface, and contact time. In the case of granules, tainted wine can be passed through the polymer particles, and the optimal rate should be applied. Other plastic items such as wine cask bladders and polypropylene lids also have scalping effects on haloanisoles [46]. A limitation for the use of plastic materials to reduce the TCA content in wine is related to the simultaneous scalping of wine color and aroma compounds [102], which can lead, in particular, to the loss of floral/fruity aromas [46]. In a recent study, the application of alimentary film (confidential composition) reduced TCA content by 81–83% after 48 h of wine-film contact [103]. Checking other wine components after this treatment showed no noticeable impact either on the color of red wines or on the phenolic and tannin composition. As for wine aroma compounds, there was no effect on the woody aroma profile; however, long-chain ethyl esters (ethyl octanoate, ethyl decanoate, and ethyl dodecanoate) were significantly absorbed, by about 70–80% after 48 h. Similar effects were also observed for synthetic bottle stoppers, which demonstrated higher absorption of the mentioned ethyl esters compared with corks [112].

Cork material itself can serve as a good absorbent of TCA and other halonisoles. It was found that cork stoppers are able to reduce the TCA content in tainted bottled wine by about 50% after 3 months of storage [46]. These results were similar for corks of different qualities, including agglomerated stoppers. Obviously, in order to reduce the TCA content in wine, corks should not be initially contaminated with TCA. Immersion of cork stoppers in tainted wine (soaking) can remove even more TCA, about 80–90% [46]. This idea has already been discussed in the previous section about the analysis of *releasable TCA*.

Subsequent works on the development of suitable polymeric materials for the removal of TCA from wine involved the usage of polyaniline-based materials and cross-linked derivatives of polyamidoamine [104]. They demonstrated a relatively high TCA absorption (>75%) and almost no impact on phenolic compounds in wine. At the same time, more research is required on the scalping of aroma compounds by these polymers. In order to eliminate tainted compounds selectively, the application of molecularly imprinted polymers (MIPs) was proposed. Tests with absorbents

Methods/absorbents used	TCA removal efficiency	Remarks	References
Polyethylene (PE) film	> 90%	• Absorption of other haloanisoles was also studied: 2,4-DCA, 2,6-DCA, TeCA, PCA • Scalping of some wine aroma compounds	[46]
UHMW PE granules	> 90%	• Some changes in color and flavor of wine	[102]
Alimentary film	81–83%	• Phenolic, tannin and color composition of the wine was stable • Concentration of woody aromas was not affected, but long-chain ethyl esters content was considerably reduced	[103]
Cork (bottled wine with corks)	~ 50%	• Absorption of other haloanisoles was also studied: 2,4-DCA, 2,6-DCA, TeCA, PCA (more chlorine atoms in haloanisole—higher absorption by cork)	[46]
Cork (soaking of corks in wine)	~ 90%		
Polyaniline- and polyamidoamine-based polymers	> 75%	• Low affinity for wine phenolic substances, but limited information on scalping of aroma compounds	[104]
Molecularly imprinted polymers (MIPs)	> 99%	• Intense absorption of other wine aroma molecules	[105]
Activated charcoal	High	• Suitable only for minorly tainted wines • High doses substantial diminish wine aromas	[27]
Zeolite	> 90%	• Zeolite integrated into filter sheets • It can reduce TCA content below its sensory thresholds (1.1–1.2 ng/L) and eliminate TBA • Allowed according to OIV and European Parliament regulations	[106–109]
Yeast hulls	27%	• Moderate reduction of TCA, but higher for other haloanisoles (55% for TeCA, 73% for PCA) • Color composition is stable. Effect on wine aroma is to be studied	[110]
Grape seed oil and milk products	• Analysis of haloanisoles after 7-days of treatment showed the following efficiency in removing pollutants: • oil > plastic film > cork > milk products		[111]
Wine blending	• Not recommended, but can be used for wines with minor TCA taint • Risk of contamination of a larger volume of wine		—

Table 3.
Methods and materials proposed for the treatment of TCA-contaminated wines.

of this type allowed the removal of TCA with a very high efficiency, >99% [105]. Simultaneously, it also revealed high retention properties toward other molecules such as 4-ethylphenol, 4-ethylguaiacol, oak lactones, 2-phenylethyl acetate, etc. Therefore, succeeding research on the absorption of other wine aroma compounds is also needed.

Among the inorganic materials, it was initially proposed to use activated charcoal. It demonstrated good results in TCA retention, but also low selectivity, i.e., high absorption of other wine components. Therefore, only slightly tainted wines are recommended to be treated with activated charcoal at doses, which are well below the maximum allowed levels (100 g/hL) in the EU for wine production [27, 113].

In this regard, zeolites, aluminosilicate minerals, seem to be more suitable absorbents. Zeolites possess a microporous structure, represented by a complex system of cavities (< 2 nm) and channels with a negatively charged surface. Due to these particularities, zeolites, as molecular sieves, have a good potential to interact and retain various molecules, including TCA. Zeolite powder can be directly mixed with contaminated wine [106] or integrated into filter plates that facilitates its industrial application. It was demonstrated that filtration of contaminated wines (5–20 ng/L of TCA) through such filters ("Fibrafix® TX-R") diminishes the TCA content to 1.1–1.2 ng/L (**Figure 10**), which is usually below the sensory thresholds [108]. In turn, in the wines contaminated with TBA (5–20 ng/L), undetectable levels of the pollutant were found after the treatment. Filtration through "Fibrafix® TX-R" plates had no significant impact on the analyzed wine aroma compounds (mainly secondary, fermentation aromas). At the same time, sensory panelists were able to distinguish between the wines filtered through the zeolite filter and a conventional filter, but no preference was given to any of the wines. As for the migration of aluminum ions from the filter sheet into the wine, it was insignificant, maximum 0.4 mg/L [108]. The application of zeolite containing filters is also described in the International Oenological Codex of OIV [109], and the recent EU Regulation (2019/934) permits the wine treatment using filter sheets with Zeolites-Y (Faujasite) for the selective removal of haloanisoles [107].

One of the gentle methods of TCA absorption involves the wine treatment with yeast hulls [110]. Several doses of yeast hulls were tested: from 100 mg to 800 mg per 1 L of wine. The effect of such treatment was moderate for TCA: the average dose (400 mg/L) provided only a limited reduction of TCA by 27%. As for other haloanisoles, they were absorbed in larger amounts: 55% for TeCA and 73% for PCA. Wine color deviation was measured for the treated wines and was minor even at the maximal dose of yeast hulls: decrease of color intensity by 3.1% (sum of OD at 420, 520, and 620 nm). Further studies about the impact of yeast hulls on the wine aroma composition can be of interest.

Among biogenic products that have also been tested to diminish TCA in wine are grape seed oil and milk products [111]. The latter exhibited a limited reduction of TCA content in wine, while the treatment with grape seed oil provided even better TCA scalping properties than plastic film. This fact demonstrates the potential of various natural products as absorbents, but the sensory effect on the wine of the used products was noticeable during tastings. The practicality, costs, and compositional consistency of these biogenic absorbents should also be taken into

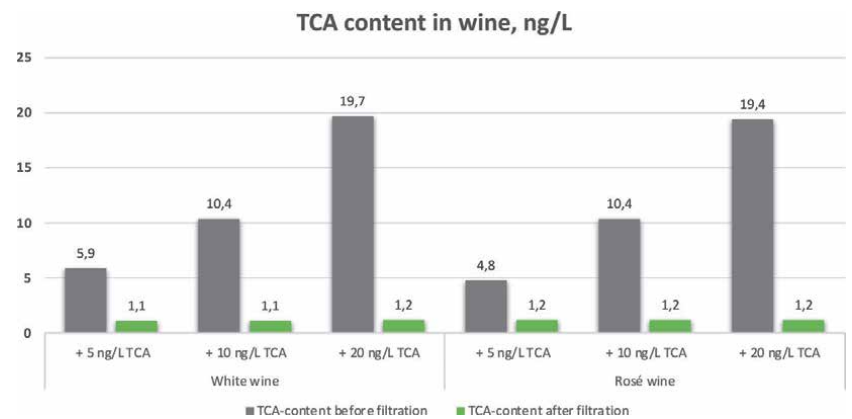

Figure 10.
Removal of TCA by wine filtration through "Fibrafix® TX-R" [108].

consideration. Moreover, the use of certain natural products may raise questions about possible allergic reactions in individuals.

Finally, the simplest, but also the most risky, method to lower TCA content in contaminated wine is to blend it with defect-free wine. This approach is not recommended and can only be accepted if the problematic wine has just a very minor TCA taint. The dilution can then reduce the TCA concentration below the sensory threshold levels. In other cases, there is a high risk that the entire volume of wine after blending will become defected.

In general, most of the methods described above are aimed primarily at large volumes of wine, while it is not yet bottled. Therefore, it is necessary to adapt these treatments to industrial scale processes, which may be less effective than test treatments on a laboratory scale. In addition, the cost efficiency of the presented treatments should be taken into account, as some of the methods can be relatively expensive.

6. Conclusions

It has been discussed in this chapter that cork stoppers are probably responsible for most of the TCA taint problems in wine. However, besides corks, TCA can also be originated from the cellar atmosphere and contaminate wines bottled with non-cork closures (screw caps, synthetic stoppers, etc). Therefore, some authors have suggested that "moldy taint" or "musty taint" may be more appropriate terms for TCA contaminated wines than "cork taint" [22].

Two main approaches to the analysis of TCA in cork stoppers have been described: determination of *total TCA* and *releasable TCA* contents. The latter is especially important for assessing the contamination of corks before wine bottling.

Then, current methods of reduction/elimination of TCA in corks were considered, which are based on two tactics: cleaning of cork material to remove TCA and sorting of corks to select "TCA-free" ones. It has been shown that application of these methods significantly reduces the incidences of TCA defects in wine nowadays. Improved cork production technologies also play an important role. They provide better control and prevention of TCA formation on the stages of bark slabs treatment, storage, etc.

For wines contaminated with TCA, methods for removing/diminishing the TCA content have been discussed (mainly industrial-scale treatments). Many of the mentioned wine cleaning methods can reduce the TCA concentration in wine by 80–90% or more, but they are not universal and not always cost-efficient. In addition, they can cause some side effects such as removal of certain positive aroma compounds.

Finally, it can be concluded that the deep understanding of the TCA problem and the further development of modern technologies give a good chance that the number of defective wines will continue to decline also in the future.

Acknowledgements

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Author details

Andrii Tarasov[1*], Miguel Cabral[2], Christophe Loisel[3], Paulo Lopes[2], Christoph Schuessler[1] and Rainer Jung[1]

1 Department of Enology, Hochschule Geisenheim University, Geisenheim, Germany

2 R&D Department, Amorim Cork, Mozelos, Portugal

3 R&D and Oenology Center, DIAM Bouchages SAS, Espace Tech Ulrich, Ceret, France

*Address all correspondence to: andrii.tarasov@hs-gm.de

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Chapter 18

New Insights about the Influence of Yeasts Autolysis on Sparkling Wines Composition and Quality

Pere Pons-Mercadé, Pol Giménez, Glòria Vilomara, Marta Conde, Antoni Cantos, Nicolas Rozès, Sergi Ferrer, Joan Miquel Canals and Fernando Zamora

Abstract

Sparkling wines elaborated using the traditional method undergo a second fermentation in the bottle. This process involves an aging time in contact with the lees, which enriches the wine in various substances, especially proteins, mannoproteins and polysaccharides, thanks to the autolysis of the yeasts. As a result of this yeast autolysis, sparkling wines benefit from better integration of carbon dioxide and a clear sensory improvement, especially in the case of long aging. This chapter synthetizes the main results that our research group has obtained about the influence of yeasts autolysis on sparkling wines composition and quality during last years, making special emphasis on the capacity of the lees to release proteins and polysaccharides as well as on their capacity to consume oxygen and thus protect the sparkling wines from oxidation.

Keywords: sparkling wines, yeast autolysis, proteins, polysaccharides, oxygen consumption

1. Introduction

In obedience to the European Regulation CE 1493/99 [1], sparkling wines differ from still wines in the level of internal pressure of carbon dioxide that must be higher than three bars. Sparkling wines are classed in the function of the CO_2 origin in two main categories: gasified wines when the carbon dioxide is from an exogenous source and natural sparkling wines when it comes from endogenous fermentation.

Gasified wines are produced simply by injecting carbon dioxide until reaching the desired internal pressure. Normally, these gasified wines have no geographical references, are very cheap and have much lower sensory quality than natural sparkling wines. Given their small interest in their sensory point of view they will not be considered in this chapter. In contrast, natural sparkling wines are obtained using a natural fermentation keeping all or a great proportion of the carbon dioxide inside the vessel in which it has been fermented.

There are different elaboration methods of natural sparkling wines depending on the type of vessel (bottle or tank), time of lees contact, the procedure of eliminating the lees, or if they have had one or two alcoholic fermentations. Moreover,

some of these natural sparkling wines are protected by *Appellations d'origine contrôlées* (AOC) such as *Champagne*, *Cava*, *Francia Corta*, *Prosecco*, *Asti*, *Crémant de Borgougne*, etc.... In that case, each AOC determines the elaboration method, authorized varieties and aging time.

Sparkling wines considered as top quality, such as *Champagne*, *Francia Corta* and *Cava*, are mainly produced by the traditional method, also called for Champagne AOC "*méthode champenoise*". The main characteristic of the traditional method is that after a first fermentation to obtain the base wine, a second fermentation, also called "*prise de mousse*", is performed inside a closed bottle [2, 3]. This second fermentation inside the bottle, and especially the aging time in contact with the lees, completely transform the sparkling wine composition and represents therefore the main differential factor regardless of other sparkling wines produced using other methods [4–6]. During the time of contact of the wine with the lees, several processes occur (**Figure 1**) that explain why the sparkling wines produced by the traditional method generally have higher quality and complexity and are much better considered by the consumers.

Briefly, once the second fermentation is completed, yeast autolysis begins [7]. Autolysis consists of the degradation process of yeast cell structures [8]. Autolysis involves the participation of hydrolytic enzymes, which, by degrading cell structures, cause the release of many substances such as amino acids, peptides, lipids, proteins, nucleotides, proteins, mannoproteins and polysaccharides [9–15]. The release of peptides, proteins, mannoproteins and polysaccharides favors the integration of carbon dioxide, which improves the perception of effervescence in the palate and increases the foam stability [6, 16]. Mannoproteins and polysaccharides also play a positive sensory role by improving mouthfeel [17], whereas some peptides and proteins can contribute to wine sweetness [18]. Some amino acids, peptides and nucleotides are also reported to participate in the umami taste [19] and to be flavor enhancers. Finally, amino acids and lipids have been described as aroma precursors [20] that contribute to the aromatic complexity of sparkling wines.

It has been also reported that yeast lees exert antioxidant activity [21] and recently it has been demonstrated the ability of the lees to consume oxygen [22]. The mechanism by which the lees consume oxygen is not clear but it could be related to the oxidation of membrane lipids [23] or with their content in glutathione [24]. Regardless of the mechanism by which lees consume oxygen, it is clear that their presence slows down the oxidative evolution of the wine by consuming the oxygen that permeates the crown cap. This oxygen consumption by lees is probably the main reason why sparkling wines can usually age for a longer time than still white wines.

In synthesis, yeast autolysis completely modifies the composition of the sparkling wine and therefore also its sensory quality. For all these reasons, the most important AOC (Appellation d'Origine Contrôlée) for sparkling wines has established minimum

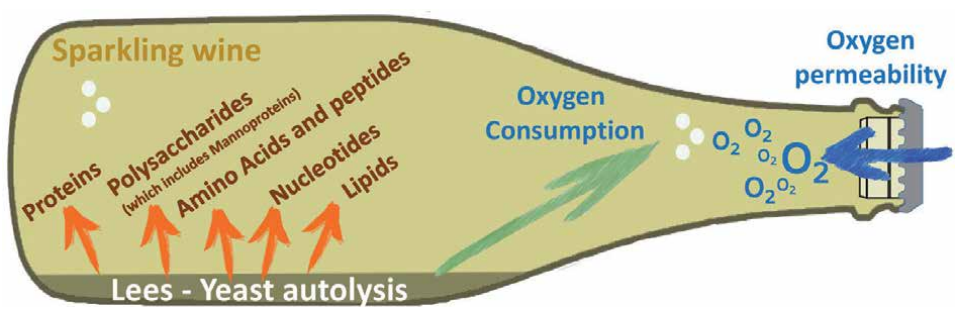

Figure 1.
Influence of the lees on sparkling wine composition.

ageing times to ensure that autolysis exerts an effect on their composition and quality. For the AOC Cava, the minimum ageing time is 9 months, though its premium sparkling wines are usually aged for longer. The AOC Cava contains two other categories of sparkling wines with extended ageing times. These are the Reserva and Gran Reserva, whose minimum ageing times are 15 and 30 months, respectively. Certain prestigious wineries produce Cavas with an even longer ageing time.

It appears, therefore, that autolysis favors the quality of sparkling wines, at least during the first few years. However, other phenomena take place in parallel—such as aromatic and color oxidation or an excessive lees flavor—which can damage the sensory qualities of these wines [22]. Therefore, we can ask ourselves until what time of aging the quality of the product is favored.

The chapter aims is to synthesize the main results that our research group has obtained on the influence of yeast autolysis on the composition and quality of sparkling wines. This study was carried out studying nine consecutive vintages and was developed in the PhD thesis of Pere Pons entitled "Yeasts autolysis on the manufacture of sparkling wines; influence of aging time on the release of polysaccharides and proteins and the consumption of oxygen by the lees" [25] that was part of the projects GLOBALVITI (global solution to improve wine production against climate change based on robotics, IT technology and biotechnological strategies and vineyard management) and CAVAWINNER (Study and Technological Improvement of the Traditional Processes for the Production of Cava) funded by the Spanish Centre for the Development of Industrial Technology (CDTI - CIEN program). To our knowledge, this is the longest time ever studied about sparkling wines from the AOC Cava.

2. Materials and methods

Figure 2 illustrates the experimental design. Briefly, this study was carried out using sparkling wines from nine consecutive vintages (2008–2016) from the Juve & Camps winery (AOC Cava, Sant Sadurní d'Anoia, Barcelona, Spain). All these

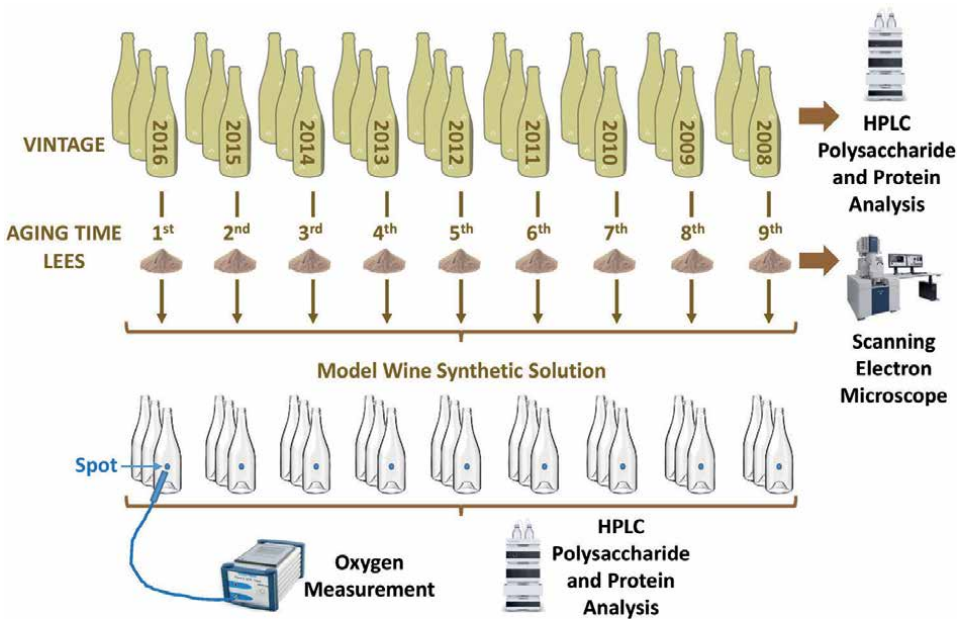

Figure 2.
Experimental design.

sparkling wines were produced with grapes from the same vineyards and were elaborated as similarly as possible. The youngest sparkling wine (2016) was disgorged 3 months after "tirage" and the sparkling wines from the other vintages were also disgorged 3 months after having completed 1–8 years of aging, respectively. In all the cases the lees were recovered, washed, resuspended in a model wine solution and bottled for subsequent analysis. These bottles were inserted with a pill for measuring dissolved oxygen by luminescence (Nomasense TM O2 Trace Oxygen Analyzer). Aliquots of the lees from the nine consecutive vintages were also used for ultrastructural observation using scanning electron microscopy [15]. Consequently, this study was performed with sparkling wines and lees from the first to the ninth years of aging.

The sparkling wines were used for color [26], polysaccharides [27] and proteins [28] analysis, for measuring the foaming properties [29] and for tasting. In parallel, the oxygen concentration was measured periodically in the bottles in which the lees were transferred [22]. Exactly 1 year later the solution was centrifuged and used for polysaccharides and protein analysis [27, 28].

3. Results and discussion

Table 1 shows the CIELab coordinates of the sparkling wines. As expected, the blue-yellow CIELab component (b*) clearly increased as the aging time increased. These data confirm a fact that is well known by winemakers: the intensity of the yellow color progressively increases over time. **Table 1** also shows the foaming properties of these sparkling wines. Both the maximal height of the foam (foamability [HM]) and the stable height of the foam (foam stability [HS]) showed a similar tendency, they increased between the first and second year of aging and decreased progressively afterward.

Figure 3 shows the polysaccharide concentration of the sparkling wines of the nine consecutive vintages. In general, no clear trend was detected either in the total concentration of polysaccharides or in any of its different fractions of different molecular weight. This lack of tendency seems to contradict what should be expected from yeast autolysis, as it should theoretically increase its concentration over time. Nevertheless, other authors also found no clear trend in the evolution of the polysaccharide fraction during the aging of sparkling wines on lees [13, 30, 31].

A possible explanation for this lack of trend maybe that polysaccharides are simultaneously released and removed from the media. Yeast autolysis may be a source of polysaccharides and mannoproteins [32]. However, polysaccharides can also disappear by precipitation [13], absorption by the riddling agents [33] and enzymatic degradation [30]. In addition, the variability among vintages may overlap making it very difficult to detect any tendency.

Figure 4 shows the protein concentration of the sparkling wines of the various vintages. Similar to what happened with polysaccharides, no clear trend was observed throughout aging time, neither in the concentration of total protein nor in any of its fractions of different molecular weight. Once again, these results may appear to contradict what is expected from yeast autolysis. However, other authors have also reported a similar erratic behavior [34–36].

Similar to what happened with polysaccharides, this lack of tendency may be related to a balance between the proteins released from yeast autolysis and those that disappear due to bentonite absorption and enzymatic degradation [9, 34, 35, 37]. Furthermore, the variability in the protein concentrations of each vintage can make it difficult to conclude.

Since no tendency was observed for either polysaccharides or proteins, it was decided to study the release of these macromolecules from the lees using a different

Aging time (years)	Lightness (L^*)				Green-red component (a^*)				Blue-yellow component (b^*)				Foamability-Hs (mm)				Foam stability-Hs (mm)			
1st	98.0	±	0.2	C	−0.88	±	0.06	CD	6.88	±	0.12	A	87	±	1	C	71	±	3	C
2nd	98.1	±	0.1	C	−1.02	±	0.04	BCD	7.69	±	0.08	B	170	±	1	E	93	±	3	E
3rd	97.9	±	0.1	C	−1.11	±	0.02	AB	8.78	±	0.07	C	110	±	5	D	81	±	1	D
4th	97.7	±	0.2	BC	−1.11	±	0.07	AB	8.66	±	0.21	C	80	±	4	BC	72	±	2	C
5th	97.4	±	0.0	B	−1.09	±	0.08	AB	9.87	±	0.13	E	76	±	4	BC	68	±	1	BC
6th	97.6	±	0.1	BC	−1.25	±	0.08	A	9.59	±	0.07	DE	61	±	1	A	53	±	3	A
7th	96.8	±	0.5	A	−1.05	±	0.03	ABC	9.39	±	0.02	DE	73	±	1	ABC	58	±	2	A
8th	97.6	±	0.1	BC	−1.00	±	0.10	BCD	10.77	±	0.19	F	71	±	4	AB	54	±	1	A
9th	97.4	±	0.2	B	−0.92	±	0.10	BCD	10.99	±	0.34	F	74	±	1	ABC	59	±	1	AB

Results are expressed and as mean ± standard deviation of three replicates. Different letters indicate the existence of statistical difference ($p < 0.05$).
Adapted from Pons-Mercadé et al. [15].

Table 1.
CIELAB coordinates and foaming properties of the sparkling wines of different aging time.

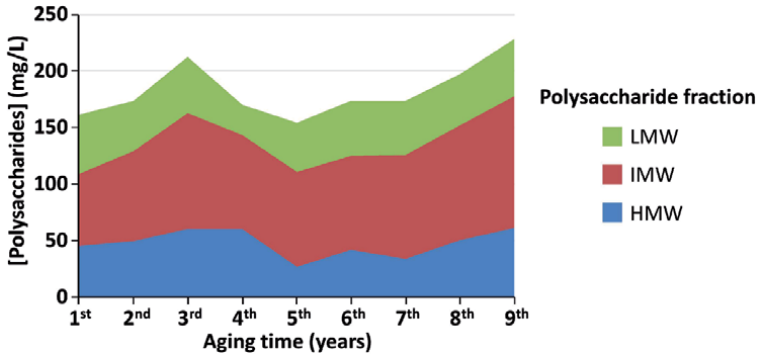

Figure 3.
Changes in the polysaccharide content of sparkling wines over ageing time. LMW, low molecular weight fraction (40–7.5 kDa); IMW, intermediate molecular weight fraction (180–40 kDa); HMW, high molecular weight fraction (>180 kDa). Adapted from Pons-Mercadé et al. [15].

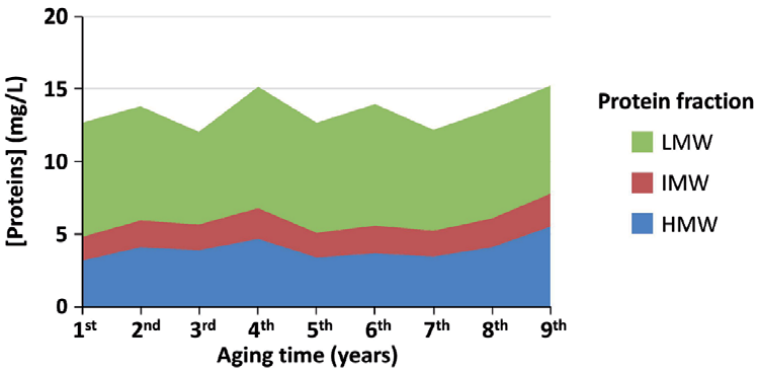

Figure 4.
Changes in the protein content of sparkling wines over ageing time. LMW, low molecular weight fraction (50–25 kDa); IMW, intermediate molecular weight fraction (75–50 kDa); HMW, high molecular weight fraction (>75 kDa). Adapted from Pons-Mercadé et al. [15].

approach consisting of analyzing the wine model solutions that had been in contact with the lees for a year. **Figure 5** shows the obtained results. The polysaccharides released in this model wine solution by the lees (**Figure 5A**) increased between the first (roughly 4 mg/L) and the second (roughly 6 mg/L) year of aging while decreasing progressively in the later vintages, reaching a minimum value in the ninth year of ageing (roughly 0.90 mg/L). The mannose concentration obtained from the hydrolysis of this polysaccharide (**Figure 5B**) showed very similar, which would confirm that they were mainly mannoproteins.

The total protein concentration released from the lees of different aging times in a model wine (**Figure 5C**) showed a similar pattern to that of the polysaccharides reaching a maximal value in the third year (roughly 0.32 mg/L) and a minimum value in the ninth year of aging (roughly 0.17 mg/L).

To reproduce the cumulative release effect of polysaccharides and proteins over the lees aging time, the concentrations of both macromolecules released from the first to the ninth year were added (**Figure 5D** and **E**). This simple approach shows a clear increase in the accumulated concentrations of both macromolecules over the ageing time. The total accumulation of polysaccharides at the end of the 9 years was 26.6 mg/L, while that of proteins was 2.4 mg/L. These values should be taken with caution since they reflect just one approach. Nevertheless, this data indicates that

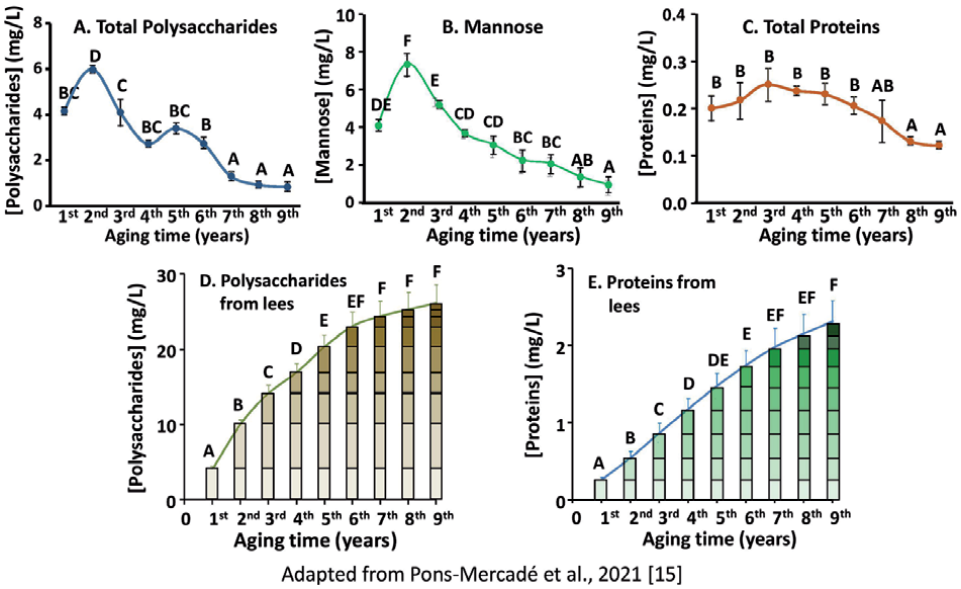

Figure 5.
Changes in the polysaccharide and protein content of sparkling wines over ageing time. A. Total polysaccharides; B. Mannose; C: Total protein; D. Polysaccharides released by lees; E: Proteins released by lees. Adapted from Pons-Mercadé et al. [15].

the release of polysaccharides and proteins from the lees during the ageing process was much lower than the usual concentrations present in these sparkling wines.

Another approach was carried out to illustrate the distribution of polysaccharides and proteins according to their origin (from the autolysis of lees or base wine) throughout the aging time. **Figure 6** shows the percentage of polysaccharides (A) and proteins (B) from lees autolysis or base wines for total concentration in the sparkling wines. This figure clearly shows that the percentage of polysaccharides and proteins from lees autolysis was extremely low in the young sparkling wines. That means that during the first year of ageing, sparkling wine had only 2% of proteins and 3% of polysaccharides from the lees. These percentages increased as

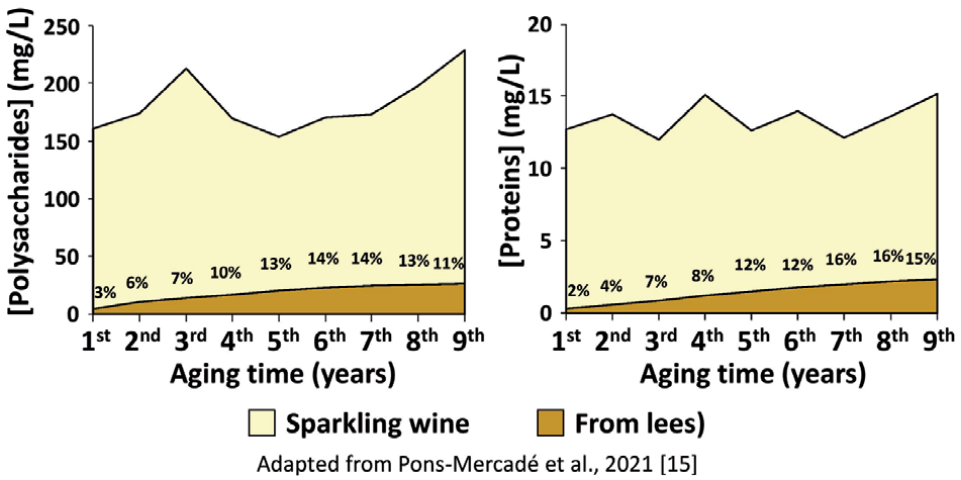

Figure 6.
Distribution of proteins and polysaccharides of sparkling wine according to their origin. Adapted from Pons-Mercadé et al. [15].

the ageing time increased and reached maximal values in the seventh year of ageing (14% for polysaccharides and 16% for proteins).

It is necessary to point out that only 88% of sparkling wines from AOC Cava are aged between 9 and 15 months and 78% of champagnes are aged between 12 and 36 months (data from the regulatory councils). Consequently, the majority of sparkling wines produced by the traditional method have percentages of polysaccharides and proteins from lees autolysis below 7%, and this value should even be lower in the youngest sparkling wines, especially those not produced by the traditional method.

As it was explained above, the bottles, in which the lees extracted from the sparkling wines were resuspended in a model wine solution, had been inserted with a pill for measuring dissolved oxygen by luminescence. The content of these bottles was saturated in oxygen and the oxygen concentration was monitored periodically for a year. **Figure 7** shows the oxygen consumption kinetics of the lees from sparkling wines from the first to the ninth year of aging time [22]. The oxygen consumption of the Control-A model wine solution (without adding lees) and the oxygen intake in Control-B (solutions without lees and oxygen) were very low and can be considered negligible (data not shown). In contrast, the oxygen consumption of all the samples containing lees increased over time, demonstrating that the lees can consume oxygen.

Moreover, this graph clearly shows that the lees of the first 3 years, especially those of the second year, consume much more oxygen than the lees of later years. It, therefore, seems clear that the ability of the lees of sparkling wines to consume oxygen increases between the first and second year and after tends to decrease throughout the aging period. The kinetic model proposed by Pascual et al. [38] was applied to these data to determine more precisely the total oxygen consumption capacity of the lees of these sparkling wines. **Figure 8** shows that the lees from the second year are capable of consuming nearly the double oxygen than those of the first or third year. Subsequently, the annual oxygen consumption decreases drastically in the older lees.

The higher oxygen consumption of the lees of the second year could be related to the described progress of the autolysis process which, according to some authors, starts slightly after 4 months and is more intense during the second year [7, 32, 39, 40]. It should also be noted that the maximal oxygen consumption-ability of the lees of the second-year match with the maximal polysaccharide and protein release and with the maximal levels of the foaming parameters [15]. All of these data seem to indicate that autolysis is at its peak during the second year of aging.

In any case, it seems that the oxygen consumption by the lees decreases drastically after 3 years of aging whereas the entrance of oxygen inside the sparkling wine

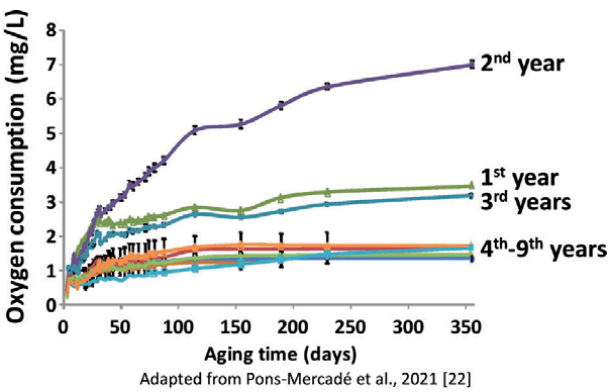

Figure 7.
Oxygen consumption by lees extracted from sparkling wines of different aging times. Adapted from Pons-Mercadé et al. [22].

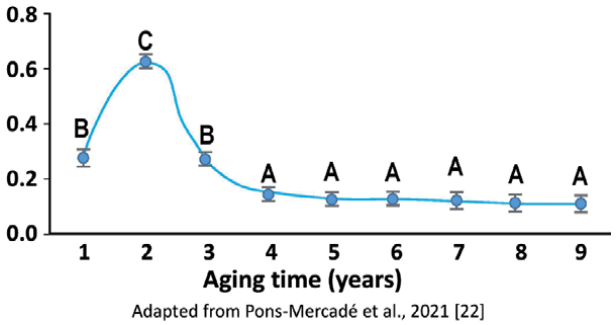

Figure 8.
Total oxygen consumed in 1 year by the lees extracted from sparkling wines of different aging times. Adapted from Pons-Mercadé et al. [22].

through the crown cap seems to be constant [41]. As long as the lees' oxygen consumption ability is greater than the oxygen permeation, the sparkling wine will be protected against oxidation. However, we can wonder what would happen when the lees stop consuming enough oxygen? When this happens, oxygen will be consumed by other wine components, especially by phenolic compounds, which will cause browning and the appearance of hydrogen peroxide that will oxidize other wine compounds in the absence of free sulfur dioxide, especially aroma compounds. Oxidation will be greater or lesser depending on the composition of the sparkling wine, which is largely dependent on the vintage and the production process.

Figure 9 try to illustrate this complex balance showing the accumulated oxygen consumption by the lees in comparison with the oxygen intake across the crown cap considering the minimal value of oxygen permeability reported by Valade et al. [41]. The comparison of the two curves is just a theoretical approximation, but even so, it provides very interesting information.

According to this approach, the oxygen permeability across the crown cap remains below the accumulated oxygen consumed by the lees during the first 3 years of aging time and exceeds it at roughly three and a half years. More exactly the interception point is at 3 years and 7 months. This data indicates that after this aging time, the oxygen consumed by the lees would not be high enough to compensate for the oxygen entrance which would probably lead to wine oxidation. It should be taken into account that this calculation was done considering the minimal value of permeability reported for crown caps and that any increase in this permeability

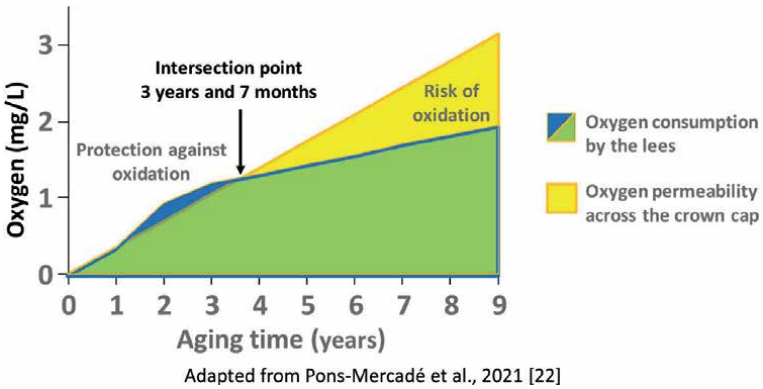

Figure 9.
Accumulation of oxygen consumed by the lees in comparison with the oxygen permeability of the crown cap. Adapted from Pons-Mercadé et al. [22].

would therefore entail an earlier point of intersection in time. For instance, with a 20% higher permeability the intersection would take place just after 2 years of aging. As aforementioned, this is only a theoretical approach based on our results but it is very useful to illustrate what happens during sparkling wine aging.

All these sparkling wines were tasted by a trained panel and the main results are synthetized in **Figure 10**. The panel was asked to blindly classify sparkling wines based on their age. The panel successfully appreciated the chronological order of these sparkling wines since established four statistically significant groups depending on their sensory perception of their aging time: Group A, which the panel considered the youngest (first year of aging); Group B (second and third year of aging); Group C (fourth to sixth years of aging) and Group D (seventh to ninth years of aging).

All panelists considered the five youngest vintages of sparkling wines as "acceptable" for consumption under their qualitative sensory criterion. However, some of them considered that after this aging time the sparkling wines were "unacceptable". These data indicate that after 5 years the sparkling wines began to be affected by excessive ageing. It should be pointed out that these sensory data match well with the previous considerations about the balance between the oxygen consumption by the lees and the oxygen permeability across the crown cap. According to these results, the oxygen consumed by the lees started to be not enough to compensate for oxygen intake through the crown cap after 3 years and 7 months of ageing. After this time, the sparkling wine does not have enough defense against oxidation. Under these conditions, its sensory quality may begin to deteriorate, though the effects of this oxidation will also depend on its chemical composition and storage conditions. In the present study, sensory deterioration seems to begin after the 5th year of aging.

Finally, some photographs of the yeasts were taken using a scanning electron microscope (SEM) [15] to visualize the yeast autolysis process in the sparkling wines aged by up to 9 years (**Figure 11**). These pictures show how the structures of the yeast cells are progressively degraded, folded and deflated. In the first image, which shows the yeast of the starter culture used for the second fermentation of the last vintage (2016), the yeast cell seems very healthy since it is elongated, ovoid and turgid without any wrinkle or folds. Several bud scars can even be identified.

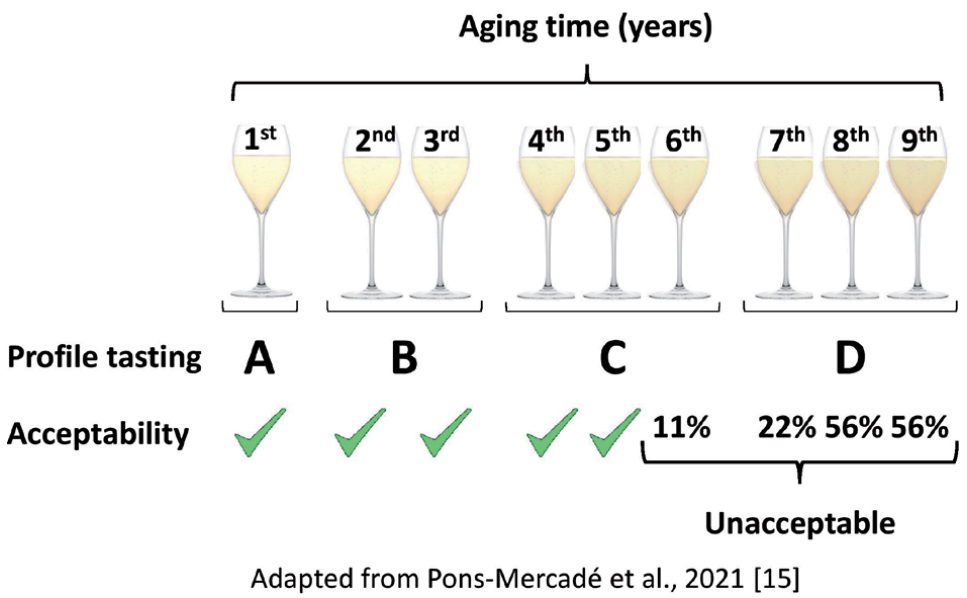

Figure 10.
Sensory analysis of the sparkling wines of the nine consecutive vintages. Adapted from Pons-Mercadé et al. [15].

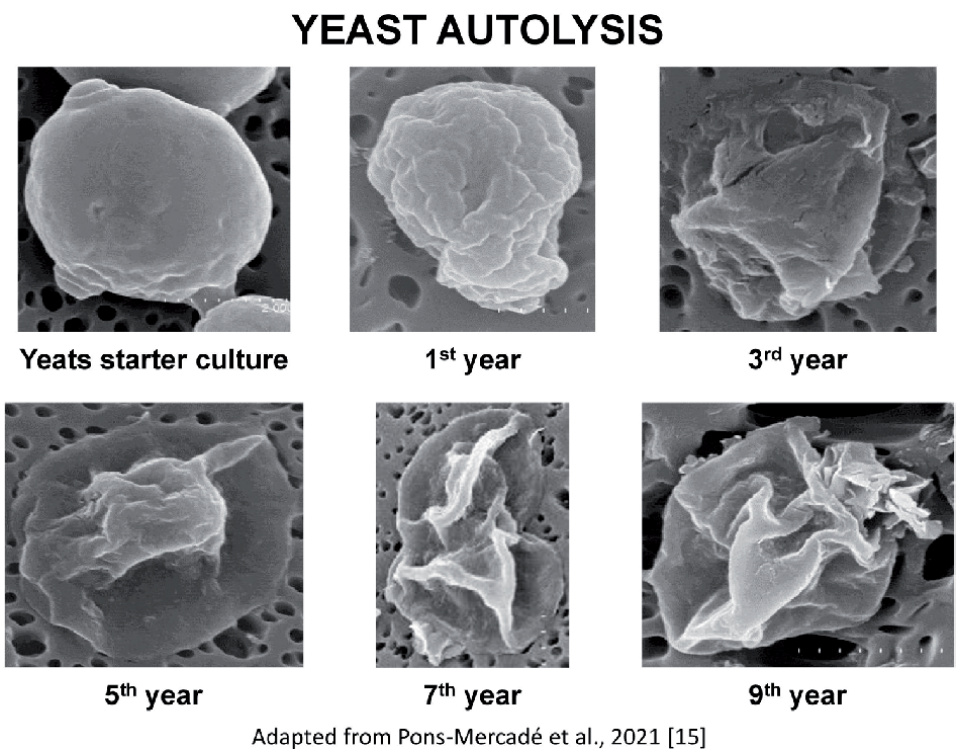

Figure 11.
Monitoring yeast autolysis overtime using scanning electron microscopy. Adapted from Pons-Mercadé et al. [15].

The second image shows what happens after the second fermentation (3 months later): the yeast cell has lost some turgor and is beginning to display wrinkles and folds. Two-years later, in the third year of ageing, the yeast cell is even more degraded and wrinkled and begins to deflate. At the fifth year of ageing, the yeast cell is completely flattened at the edges and retains only a little turgor in the middle, which is full of wrinkles and folds. In the seventh year of ageing, the yeast cell is even more degraded and deflated and the center of the cell has crumbled, wrinkled and flattened. Finally, in the ninth year, the yeast cell has completely collapsed and some of its structures are broken.

4. Conclusions

It can be concluded that the lees of sparkling wines elaborated using the traditional method have a real capacity to release proteins and polysaccharides. However, the proportion of polysaccharides and proteins from lees autolysis is very low in the young sparkling wines, roughly only 2–3% in the first year of ageing and around 7% in the third. This suggests that the real impact of polysaccharides and proteins from lees autolysis in the sparkling wines disgorged before the end of the first year should be very low. Wine producers should bear this conclusion in mind since most sparkling wines elaborated by the traditional method are aged for less than 1 year and those made by other methods are aged even less. Consequently, only sparkling wines aged for longer would therefore benefit from a greater presence of polysaccharides and proteins from yeast autolysis.

It can also be stated that lees consume oxygen and therefore they protect sparkling wine against oxidation. However, the lees' capacity to consume oxygen decreases

drastically after 3 years of aging, reaching values lower than those of the theoretical oxygen permeability of the crown cap after about 3 years and a half of aging. Producers of sparkling wines should also bear in mind because after this time the ability of the lees to protect against could not be enough. Some panelists considered that the quality of the sparkling wines was negatively affected after 5 years of aging due to excess oxidation. These data explain what AOC Cava winemakers know empirically. Only some high-quality sparkling wines made using the traditional method can age more than 3 years without being affected by oxidation, and in this case, sparkling wines reach an extraordinary level of complexity that only long aging can provide.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Author details

Pere Pons-Mercadé[1], Pol Giménez[1], Glòria Vilomara[2], Marta Conde[2], Antoni Cantos[2], Nicolas Rozès[1], Sergi Ferrer[3], Joan Miquel Canals[1] and Fernando Zamora[1*]

1 Departament de Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, Tarragona, Spain

2 Juvé and Camps SA, Sant Sadurní d'Anoia, Barcelona, Spain

3 ENOLAB, Departament de Microbiologia i Ecologia, Institut BioTecMed, Universitat de València, València, Spain

*Address all correspondence to: fernando.zamora@urv.cat

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